ARTICLE

Assessment of the cytogenetics and leaf anatomy of synthetic polyploids of *Eucalyptus* clones

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Abstract: *The induction of polyploidy in Eucalyptus can lead to higher-yielding and more adapted clones, with better wood quality. After artificial polyploidization, it is necessary to certify the effectiveness of the process through cytogenetic analyses and anatomical trait evaluations. Thus, the aim of this study was to certify the chromosome duplication in artificially polyploidized clones of Eucalyptus grandis × Eucalyptus urophylla using cytogenetic analysis, flow cytometry, and leaf anatomy measurements. Chromosomal counts and DNA content estimation allowed the identification of artificially polyploidized clones and mixoploids. Polyploid clones had larger stomata diameter than diploids. The increase in stomata size was accompanied by lower stomatal density in most polyploid clones. The typical gigas effect of polyploidized plants was demonstrated by the expressive increase in leaf area in polyploid individuals compared to diploids. Our findings indicate that the use of polyploidy in Eucalyptus breeding is promising, allowing the discrimination of diploid, tetraploid, and mixoploid plants.*

Keywords: *Eucalyptus breeding, polyploidy, chromosomal counts, flow cytometry, leaf anatomy*

INTRODUCTION

The growing demand for wood from *Eucalyptus* L'Hér, especially for pulp production, requires the production of new clones with better performance. However, as productivity levels of eucalypt clones are already high, conventional plant breeding might not provide the increases needed to meet new demands. Therefore, different strategies are being used to accelerate the process, such as genomic selection, gene editing, and polyploidization of genotypes of interest (Han et al. 2011).

Induced polyploidy has been indicated as a valuable tool for genetic breeding of economically relevant plants, as polyploid plants commonly display greater vigor and larger size. In addition to increasing productivity by exploiting the *gigas effect*, the main advantage of polyploidization in *Eucalyptus* breeding is to produce triploid sterile plants, which may exhibit more vegetative growth by redirecting resources that would be used by reproductive organs (Souza et al. 2022).

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After artificial polyploidization, it is necessary to confirm the effectiveness of the process through cytological and cytogenetic analyses. Flow cytometry is a fast and easy method for large-scale analysis to estimate DNA content (Pereira et al. 2012). Nevertheless, results are based on a sample of cells, and in some cases, the sample can show ambiguous outputs regarding the ploidy level, requiring confirmation by chromosome counts (Bustamante et al. 2015). Certification via chromosome counts, although more laborious, is a more reliable procedure, as it determines the exact number of chromosomes in individual cells (Pereira et al. 2012), allowing the identification and quantification of aneuploid and mixoploid cells with precision. Another alternative is to indirectly evaluate chromosome duplication by detecting changes in anatomical and morphological traits, such as stomata and leaf area measurements. This procedure is based on observation of increases in vigor and cell and organ sizes in polyploid plants compared to diploids, which is known as the gigas effect (Del Pozzo et al. 2015). It is also essential to evaluate the performance of polyploids obtained under field conditions, since variation among clones obtained from the polyploidization of a diploid clone is expected (Souza et al. 2022).

In this context, the aim of the present study was to certify the chromosome duplication of artificially polyploidized clones of *Eucalyptus grandis* Hill ex. Maiden *× Eucalyptus urophylla* S. T. Blake using cytogenetic analyses, flow cytometry, and leaf anatomy.

MATERIAL AND METHODS

Plant material

Clones obtained from artificial hybridizations between *E. grandis* and *E. urophylla*, provided by the Suzano SA forestry company, were evaluated considering 10 plants of each clone, five putative tetraploids (A, B, C, E, and F), and one diploid, for a total of 60 plants. These seedlings were kept in pots in a greenhouse. Tetraploid clones were obtained according to the chromosome duplication protocol established by the company, using the antimitotic agent colchicine in explant shoots.

Chromosome preparations and fluorescent in situ hybridization (FISH)

Root tips were collected and pre-treated with 0.5% (w/w) amiprophos-methyl (APM, Nihon Bayer Agrochem) and 4 µM of dimethyl sulfoxide (DMSO, Sigma-Aldrich®) for 3 hours at room temperature. The roots were then fixed in a methanol: acetic acid solution (3:1) for 24 hours and stored in 90% ethanol at -20 °C.

Cell wall digestion was performed in an enzyme mix (2.5% pectinase Sigma-Aldrich® + 2.5% cellulase Sigma-Aldrich® + 2.5% pectolyase Sigma-Aldrich®) for 2 h 45 min, at 37 ºC. Slides were prepared using the cell dissociation technique (Carvalho and Saraiva 1993) and air dried.

Slides were aged for seven days and subjected to the CMA/DAPI fluorescent banding procedure (Guerra and Souza 2002), with a few modifications. The slides were initially mounted with 40 µL of McIlvaine buffer pH 7.0 for 20 minutes, followed by 20 µL of chromomycin A (0.5 mg mL^{-1}) with $MgCl_2$ 2.5 M for 1 hour. Chromosomes were counterstained with DAPI (1.5 µg mL^{-1}) in 9 µL of Vectashield. The slides were stored at 4 ºC for at least three days prior to evaluation.

Sequences of 35S rDNA (pTa 71 *Triticum aestivum* L.) and telomeric probes, labeled by nick translation and PCR, respectively, with biotin and digoxigenin, were used as probes for FISH. A hybridization mixture containing 50% formamide, 2 × SSC, 10% dextran sulfate, and approximately 40 ng mL^{-1} of the 35S and telomeric probes was applied on the chromosome preparations. Slides were placed on a hotplate at 80 °C for 3 min to denature the chromosomes/probes and were then kept in a humid chamber overnight at 37 °C (Křivánková et al. 2017). Post-hybridization washes were carried out according to Masoudi-Nejad et al. (2002). Probes were detected with anti-dig and anti-biotin antibodies, using tris/sodium chloride (TNB) buffer for 1 h at 37 °C. The slides were evaluated in an Olympus BX60 epifluorescence microscope with the excitation/emission wavelength of 358/461 for DAPI, 490/525 for CMA and FITC, and 550/575 for TRITC. Chromosome counts were performed on 30 metaphases per clone.

Estimation of DNA content

DNA content was estimated by flow cytometry using leaf tissue, according to Doležel and Bartoš (2005), with some adaptations. Approximately 20-30 mg of young leaves were used per sample. Three replicates were performed for each

plant. The amount of DNA of *Solanum Lycopersicon* L. 'Stupické' was used as a standard reference (2C = 1.96 pg) (Doležel et al. 1992). Samples were macerated in a Petri dish containing 1 mL of cold woody plant buffer (WPB) (Loureiro et al. 2007) to obtain a nuclear suspension, to which 25 µL propidium iodide (1 mg mL^{-1}) was added. At least 10,000 nuclei were analyzed per sample. Histograms with coefficients of variation under 0.8% were obtained using a FACS Calibur cytometer (Becton Dickinson) and the Cell Quest software (Becton, Dickinson and Company, San Jose, CA, USA). The mean nuclear DNA content was estimated with the WinMDI 2.8 (2009) software. Based on the mean values of nuclear DNA content, clones were grouped by ploidy level within each genotype, and analysis of variance and the Scott-Knott test (1974) were performed ($p < 0.05$) using the R software (R Core Team 2020).

Stomata analysis and leaf area

Leaves from *Eucalyptus* clones were collected from the fourth stem node, counting from the apex of the plant, on day 140. The collections were fixed in FAA solution (90% ethanol, 5% formaldehyde, and 5% acetic acid) for 72 hours at room temperature and stored in 50% ethanol (Johansen 1940).

Paradermic sections were taken, and semipermanent slides were mounted with glycerin medium. Observations were made in a light microscope equipped with a camera, using the Axio Vision software under a total magnification of 400x and real visual field of 0.04843 mm^2.

In statistical analysis, eight leaves of each genotype, two fragments per leaf, and five fields of the abaxial epidermis per fragment were evaluated. In each field, three stomata were randomly selected for measurements of polar diameter (PD) and equatorial diameter (ED). Stomatal density (SD - number of stomata per unit area) and stomatal functionality (SF - polar diameter/equatorial stomata diameter ratio) were estimated (Ambrósio et al. 2020).

Leaf area was measured in plants of 210 days of age, randomly selected in each treatment. Five leaves from the fourth node below the plant apex were collected from each plant sampled. The leaves were digitalized using a manual scanner and evaluated on ImageJ software (Schneider et al. 2012).

Analysis of variance was performed using the leaf area and stomata measurements, and means were compared by the Scott-Knott test (1974) using the R software (R Core Team 2020).

RESULTS AND DISCUSSION

The cytogenetic analyses confirmed metaphases with 44 chromosomes and the occurrence of mixoploids, with chromosome numbers ranging from 22 to 44 in plants treated with the same polyploidy inducer (Figures 1 and 2A). This variation demonstrated the occurrence of mixoploidy (Figures 1, 2B and 2C). All metaphases from the F clone of *E. grandis × E. urophylla* exhibited 2n = 44 chromosomes. In the remaining polyploid clones, cells with 22, 27, 30, and 40 chromosomes were also observed. The chromosome number and morphology observed in diploid plants are consistent with previous descriptions, invariably reporting 2n = 22 chromosomes for *Eucalyptus* species (Haque 1984, Matsumoto

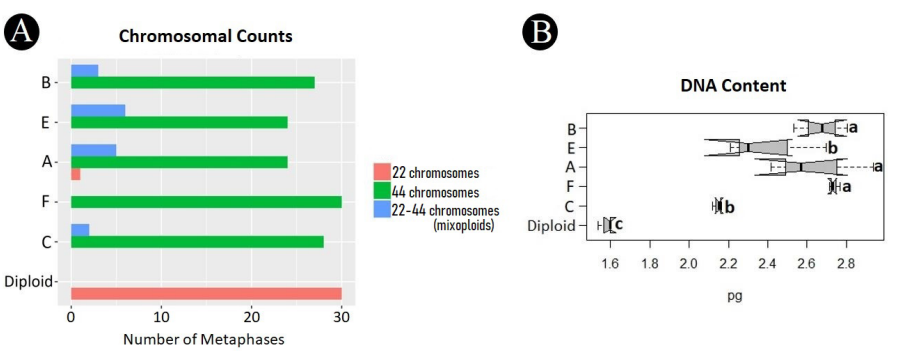

Figure 1. A. Chromosomal counts and number of metaphases evaluated of *E. grandis* x *E. urophylla*, diploid and polyploid (A, B, C, E, and F) clones. B. DNA content (2C value) in picograms (pg) of the same clones. Means followed by the same letter belong to same group according to the *Scott Knott* (1974)' test, at the probability of 5%.

et al. 2000, Bachir and Abdellah 2006) and symmetric karyotypes (Matsumoto et al. 2000). According to Haque (1984), this consistency and reduced number of chromosomes may explain the ease with which hybrids between eucalypt species arise in nature and the fertility they exhibit.

The diploid clone, used as a control, invariably showed 22 chromosomes. Many metaphases from diploid and polyploidized clones displayed supposed chromosomal fragments resulting from breaks or gaps in some chromosomes (Figures 2D, 2E, and 2F). These presumed fragments were differentiated by their size and morphology in comparison with intact chromosomes. Break or gap sites were identified by CMA+ bands (regions rich in CG) (Figure 2E), colocalized with 35S rDNA sites, as shown by FISH (Figure 2F). The telomeric probe did not show fluorescent labeling in one of the terminal regions, confirming that the fragment is the detached nucleolus organizer region (NOR) segment (Figure 2F).

The position of the CMA+ bands, colocalized with 35S rDNA sites in two chromosomes of the diploid *E. grandis* × *E. urophylla* (2n = 2x = 22) clone was also previously observed by Ribeiro et al. (2016). This site constitutes the NOR and often detaches from the chromosomes due to extension and late condensation, but it generally does not promote fragmentation and loss of DNA (Flavell et al. 1988, Gustafson et al. 1988, Sakowicz and Olszewska 1997). The presence of gaps and extended ribosomal sites in metaphasic chromosomes was also observed in other species, such as *Jatropha curcas* (Gong et al. 2013), *Citrus sinensis* (Lan et al. 2016), *Lolium multiflorum* (Rocha et al. 2017), and *Festuca arundinacea* (Ferreira et al. 2018). The nature of extended 35S rDNA sites may complicate chromosome counts, especially in species with small chromosomes and a symmetric karyotype, as in *Eucalyptus*. Oudjehih and Abdellah (2006) reported the occurrence of chromosomal breaks or gaps leading to incorrect chromosome counts in some eucalypt species, which may be a problem in identifying mixoploid plants. This problem can be overcome by analyzing many metaphases, observing the size of the chromosomes, and using CMA banding or FISH rDNA 35S. Molecular techniques like FISH help avoid mistaken chromosome counts, and they even elucidate chromosome pairs (Ribeiro et al. 2016).

Mixoploidy may occur in artificially polyploidized *Eucalyptus* plants because antimitotic agents act on explant cells at different cell cycle stages (Pereira et al. 2012). A study by Lin et al. (2010) reported that despite achieving a high rate of polyploidy induction in *E. globulus* with colchicine, the occurrence of mixoploid individuals remained an obstacle throughout the process. Similar results were recently obtained using different polyploidy inducers in two *Eucalyptus* species. Tetraploid and mixoploid plants were generated using trifluralin and oryzalin on *E. urophylla* clones (Moura et al. 2020), and colchicine on *Eucalyptus dunnii* Maiden clones (Castillo et al. 2020).

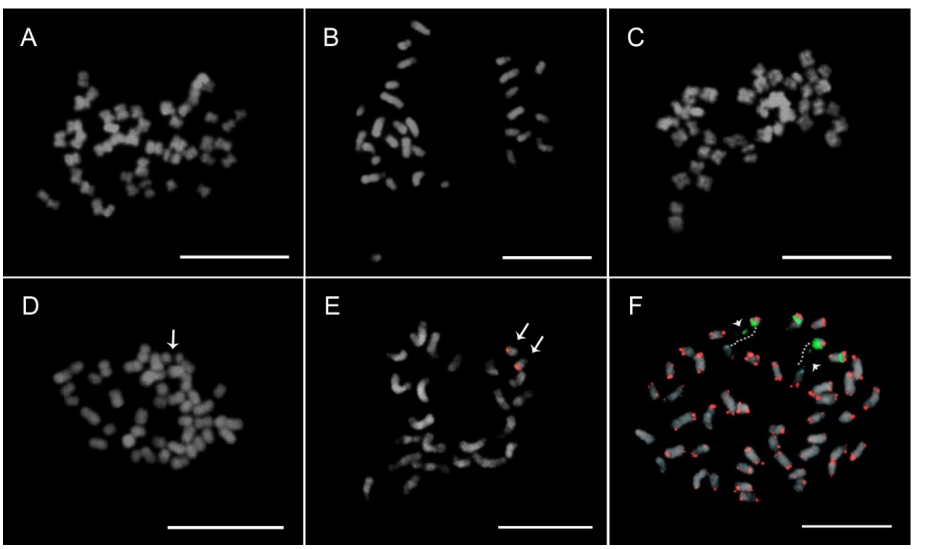

Figure 2. Mitotic metaphases of *E. grandis* x *E. urophylla* clones. Polyploid clones: A. 44 chromosomes; B. 30 chromosomes; C. 40 chromosomes; D. 44 chromosomes + 1 detached NOR segment (arrow). E. Diploid clones: 22 chromosomes + 2 detached NOR segment (arrows) and CMA bands (orange fluorescence) co-located with 35S rDNA sites (green). F. Polyploid clone: 44 chromosomes + 2 detached NOR segment, 35S rDNA sites (green fluorescence and white dotted line indicate chromatin fibers weakly marked) and telomeric sequences (red fluorescence). Bar: 10 µm.

Variation in chromosome number - mixoploidy - is not desirable, since it may compromise the fertility and uniformity of plants, depending on its extension (Pereira et al. 2012), though the use of mixoploid plants in the field can be feasible. In addition, under field conditions, variation among clones obtained from the polyploidization of a diploid is expected (Souza et al. 2022), since mixoploidy is a common phenomenon in artificial polyploidization protocols (Julião et al. 2020).

The estimated nuclear DNA content was 1.57 pg for the diploid clone and 2.15-2.74 pg for the polyploids. They were clustered in two statistically different groups: one containing clones A, B, and F, and the other with clones C and E (Figures 1 and 2). This DNA content difference among polyploids is consistent with the mixoploidy identified in the chromosome counts. The DNA content estimates in diploid plants of *E. grandis* × *E. urophylla* found in our study (1.57 pg) differ by 0.4-0.5 pg from descriptions of other diploids, such as *E. globulus* (1.13 pg) (Marie and Brown 1993), and *E. globulus, E. grandis*, and *E. urophylla* (1.05, 1.09, and 1.01 pg, respectively) (Praça et al. 2009). These differences may be due to different technical factors, such as calibration of the cytometer and differences in the reference standard, plant material, extraction buffer, and fluorochrome used (Timbó et al. 2012).

Changes in phenotypic expression among ploidy levels were observed, especially in stomatal diameter. Polyploids had larger stomatal diameters (Figure 3). In some cases, there were no significant differences between diploids and polyploids (Figure 3). This distinction in stomata size between diploid and polyploid genotypes was similar to results reported for *E. polybractea* (Fernando et al. 2019), in which the mean stomatal diameter was larger in tetraploids than in diploids. Both studies confirm that stomatal analysis can be used indirectly to identify ploidy levels in *Eucalyptus*, which has also been shown for other plants (Omidbaigi et al. 2012, Feng et al. 2017, Chaves et al. 2018).

The increase in stomata size was accompanied by lower stomatal density in most polyploid clones (Figure 2). Diploid plants exhibited a larger stomatal density, although statistically, they were similar to polyploid clones B and C (Figure 2). The smaller number of stomata per mm² in tetraploid plants than in diploid plants was also observed in *E. urophylla* (Moura et al. 2020). Stomata had an ellipsoid shape in all clones, as the equatorial diameter was smaller than the polar diameter (Figure 2). Regarding stomatal functionality (polar diameter/equatorial diameter), the diploid clones had larger values (2.65), which were statistically different from polyploids (Figure 3).

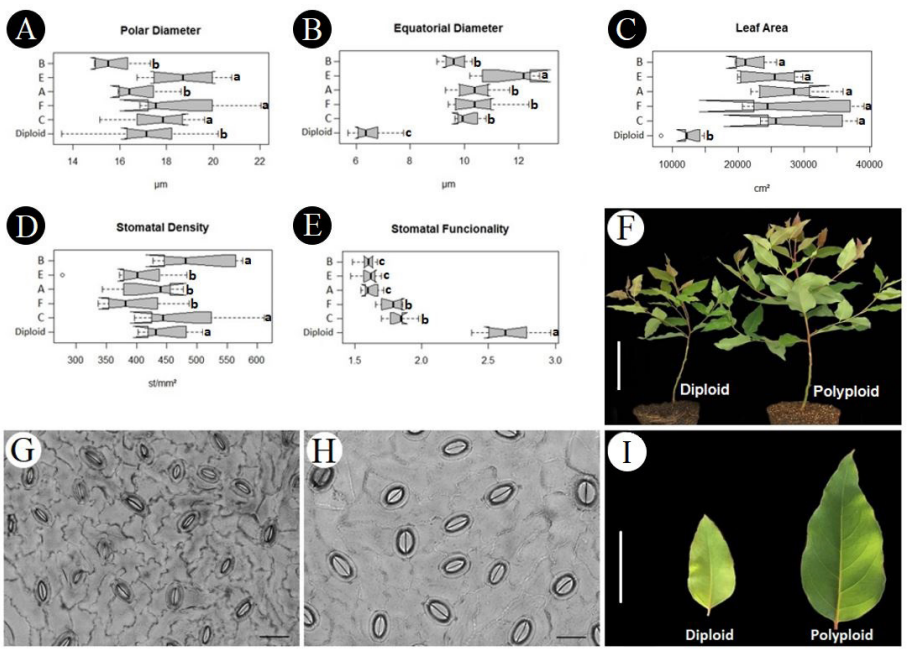

Figure 3. Polar diameter – PD (A), equatorial diameter – ED (B), leaf area (C), stomatal density – SD (D), and stomatal functionality – SF (E) of the abaxial leaves face of diploid and polyploid *E. grandis* x *E. urophylla* clones. Means followed by the same letter belong to same group according to the *Scott Knott* (1974)' test, at the probability of 5%. Paradermic sections of the abaxial leaves face from diploid (G) and polyploid (H) clones (Bar: 50 µm), leaves of 210 days after emergence (I) (Bar: 5 cm) and plants at 135 days (F) (Bar: 15 cm).

An increase in DNA content may affect the size of vegetative organs and alter tissue structure (Hodgson et al. 2010, Balao et al. 2011, Del Pozzo and Ramirez-Parra 2015). However, our findings suggest that the more elliptical shape of the stomata in diploid plants was advantageous for stomata functionality. In that sense, it is assumed that diploid plants can be more efficient in opening and closing the stomata, reducing water loss through transpiration. According to Khan et al. (2003), stomata shape is an adaptive trait for plants, since the ellipsoid shape has a smaller equatorial diameter, which results in increased functionality. However, polyploids tend to lose more water due to their larger stomata. In that case, mechanisms for stomata opening and closing during transpiration are slower. Changes in stomatal conductance due to larger stomata and lower density can also increase susceptibility to water stress (Fernando et al. 2019).

The typical gigas effect of polyploidized plants was demonstrated by the expressive increase in leaf area in polyploid individuals compared to diploid individuals (Figure 3). The trait increased significantly according to ploidy levels (Figure 3), with averages of 26,636.76 cm² for polyploids and 12,313.07 cm² for diploids. These values represent an increase of 46% for polyploidized clones (Figure 3). Likewise, Fernando et al. (2019) found an increase in leaf structure and area in tetraploid plants of *E. polybractea* compared to diploids.

Polyploidy plays a crucial role in tree breeding and can produce new phenotypes for various purposes. One of the goals of chromosome duplication is to obtain larger cells and, consequently, higher yields. Polyploid plants can also improve the quality of eucalyptus wood, enhancing its suitability for pulp and paper production (Souza et al. 2021). Furthermore, successful breeding of diploids and tetraploids in the field will pave the way for triploid breeding and sterile seed production, an attractive possibility for the industry. Polyploid research in *Eucalyptus* is still an open field for further studies, and the current results indicate that it is an important approach for tree breeding.

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