Induction and evaluation of tetraploid plants of *Eucalyptus urophylla* clones

Luciana Coelho de Moura[1], Aloisio Xavier[2], Lyderson Facio Viccini[3], Diego Silva Batista[4,*], Elyabe Monteiro de Matos[5], Ricardo Gallo[5], Brígida Maria dos Reis Teixeira[6], Wagner Campos Otoni[7]

[1]Faculdade de Engenharia Florestal, Universidade Federal do Mato Grosso, Cuiabá, MT, 78085-100, Brazil
[2]Departamento de Engenharia Florestal, Universidade Federal de Viçosa, Viçosa, MG, Brazil
[3]Departamento de Biologia, Laborátorio de Genética e Biotecnologia, Universidade Federal de Juiz de Fora, Juiz de Fora, MG, Brazil
[4]Departamento de Agricultura, Universidade Federal da Paraíba, Campus III, Bananeiras, Paraíba, Brazil
[5]Departamento de Ciência Florestal, Universidade Federal Rural de Pernambuco, Recife, PE, 52171-900, Brazil
[6]CMPC, Rua São Geraldo, 1680, Bairro Ermo, CEP 92703-470, Guaíba, RS, Brasil
[7]Laboratório de Cultura de Tecidos/BIOAGRO, Departamento de Biologia Vegetal, Universidade Federal de Viçosa, Av. P.H. Rolfs, s/n, 36570-900 Viçosa, MG, Brazil

[*]Corresponding author: diegoesperanca@gmail.com

Abstract

Eucalyptus urophylla is an important species of the genus *Eucalyptus* that is used across the world, especially in Brazil. It is used for pulp and paper production, and charcoal production for iron and steel industry, thus making the development of biotechnological approaches essential for the breeding programs of this species. The polyploidy induction is one such approach that may introduce phenotypic characteristics of interest to the market and industry. This is the first study that shows the production, evaluation, and development of tetraploids (4x = 44) from a clone of *E. urophylla.* For this, the shoot apical meristems were cultured for elongation using *in vitro* conditions for 15 with 5 or 10 µM colchicine, trifluralin, or oryzalin. Following this, every 30 days, the elongated plantlets were transferred to JADS medium for multiplication using tufts as explants. Thereafter, four tufts containing six to eight buds each were inoculated in flasks followed by six subcultures and were then transferred to the greenhouse for acclimatization. The experiment was performed in a completely randomized design in a factorial scheme (3x2+1: antimitotic substance x concentrations + control), with 12 replicates. Analysis of nuclear DNA content and chromosome counting by flow cytometry, photosynthetic pigment quantification, stomatal size and density, survival, and rooting were performed for 110 days after acclimatization. Data were submitted for analysis of variance followed by Scott & Knott's test ($P \leq 0.05$). As a result, we observed that the oryzalin and trifluralin treatments were efficient in the induction of tetraploid plants, while colchicine proved to be ineffective. Also, trifluralin treatment at 5 µM provided a higher polyploidy induction (18.2% tetraploids) in comparison to the others. Diploid plants displayed 1.41 pg of nuclear DNA content with 22 chromosomes, while tetraploid plants showed 2.86 pg with 44 chromosomes. In conclusion, the tetraploid plants, as compared to the diploids (control), were found to show higher chlorophyll content, larger but fewer stomata, and lower rooting and survival, which can be mitigated nonetheless through silvicultural techniques. This study shall bring new perspectives for the breeding programs of *E. urophylla*.

Keywords: Chromosome doubling, *Eucalyptus*, micropropagation, polyploids, stomatal density.
Abbreviations: BAP_6-benzylaminopurine, BIOAGRO_ Institute of Biotechnology Applied to Agriculture, DMSO_ dimethyl sulfoxide, IBA_ Indole-3-butyric acid, NAA_naphthaleneacetic, PVP_polyvinylpyrrolidone

Introduction

There are several factors that are responsible for the production and increased plantation of *Eucalyptus* worldwide. Special importance must be given to the implementation of breeding programs, the production of hybrid and elite clones, and advances in the knowledge on vegetative propagation and biotechnology approaches (Xavier et al., 2013).

The use of biotechnological methods aiming to enhance the production of Brazilian forests is increasing. Among these methods, the induction of polyploid plants is attracting the attention of producers of fruits and ornamental species, as well as the forest companies (Sattler et al., 2016).

"Polyploidy" is a hereditary condition characterized by having more than two complete sets of chromosomes in the genome, which might increase the size of certain plant organs constituting in many cases an economically valuable product (Majdi et al., 2010; Sattler et al., 2016).

Many commercially important polyploid cultivars have been produced with several desirable agronomical traits such as increased fruit size, greater productivity in vigorous plants, higher disease resistance, and reduction or absence of fruit seeds in the plants (Grosser and Chandler, 2004; Gu et al., 2005; Reforgiato et al., 2005; Yang et al., 2006; Isuzugawa et al., 2014).

Polyploidy induction resulting in the plants with larger vigorous flowering and, developed showy branches have been used to increase the production of biomass of the phytochemicals (Dehghn et al., 2012; Xu et al., 2013; Hsia et al., 2013; Gomes et al., 2014; Corrêa et al., 2016), thereby proving them to be economically valuable in the market (Escadón et al., 2007; Aversano et al., 2012).

Among the forest species, the synthetic tetraploids of *Acacia mangium* have been used earlier in Vietnam for the production of triploids that showed a significant increase in growth, yield of kraft pulp, and lengths of wood fiber in comparison to its other progenies with different ploidy (Chi et al., 2015). It has also been previously reported that induced tetraploids of the *Hymenaea courbaril* (commonly known as plant jatobá) showed larger stomata in comparison to its diploid progenies (Bona et al. 2016). Therefore, synthetic triploids and sterile individuals constitute interesting applications for polyploidy induction (Griffiths et al., 2000). Triploids are generally used in fruit breeding to obtain seedless fruits (Cuenca et al., 2015; Navarro et al., 2015), as well as to control invasive plants (Brand et al., 2016). Similarly, in *Eucalyptus*, triploid plants can also be used, for avoiding pollen contamination from other transgenic plants, a phenomenon known as gene introgression (Liu et al., 2013).

Antimitotic compounds such as colchicine, oryzalin, and trifluralin have been used earlier to duplicate chromosome numbers in the plants. Colchicine has been successfully applied in species *viz.*, *Xanthosoma sagittifolium* (Oumar et al., 2011), *Catharanthus roseus* (Xing et al., 2011) and, *Eriobotrya japonica* (Blasco et al., 2015). Similarly, polyploidy induction with oryzalin was employed in *Smallanthus sonchifolius* (Viehmannova et al., 2009), and *Solanum* spp. (Greplova et al., 2009). In addition, trifluralin was used in *Gaura lindheimere* (Pietsch and Anderson, 2006) and *Hymenaea courbaril* (Bona et al., 2016).

Nevertheless, the most efficient method of polyploid induction and its benefits in *Eucalyptus* is still unknown. Although, considering the commercial interest of this genus and other advantages observed in several plant species, the development of polyploidy induction methods and behavior of polyploid clones in *Eucalyptus* species is a field of great interest. Therefore, in the present study, we developed a protocol for the successful production of polyploid clones of *Eucalyptus urophylla* and evaluated their development and stability.

Results

Effect of trifluralin, oryzalin and colchicine application on the induction of polyploidy

The explants used in our polyploidy treatments were found to survive, that indicated that the concentrations used for these antimitotic agents were non-toxic for *Eucalyptus urophylla*.

Further, tetraploids (4x) and mixoploids (2x + 4x) plants were obtained from the *Eucalyptus urophylla* clone using trifluralin and oryzalin as antimitotic agents. The treatment using trifluralin at 5 μM provided the highest percentage (18.2%) of tetraploid plants at all the levels of evaluations (Table 1). However, colchicine proved to be inefficient for the polyploidy induction in *Eucalyptus urophylla*.

The induced tetraploid plants were found to be stable during the events, E3, E4, E6, E8, and E12, and remained as

tetraploids, even after performing three evaluations (Table 1). On the other hand, the treatment with 5 μM trifluralin helped in inducing the most stable tetraploid events, E3 and E4. On the contrary, the mixoploid events, E4, E5, E7, E9, E10, and E11 as expected, remained unstable, displaying variations in the ploidy along with the leaves and production of diploid or tetraploid cells within the same individuals.

Nuclear DNA content and chromosome counts showed that diploids of *Eucalyptus urophylla* had, on an average, 1.41 pg of DNA and 22 chromosomes, while tetraploid plants displayed, on an average, 2.86 pg DNA and 44 chromosomes (Figures 1A, 1B, 1C, and 1D). The value of the coefficient of variation was observed to be 5% lower than expected, thereby assuring the accuracy of the DNA content measurements (Dolezel et al., 1989).

Significant differences were also observed in photosynthetic pigments in *ex-vitro* acclimatized plantlets. The chlorophyll *a* content in the tetraploid events E3 and E4, both under trifluralin 5 μM treatment, formed a different group of the E1 diploid event, and the other events analyzed (Table 2). For chlorophyll *b*, carotenoids, and total chlorophyll events E1, E3, E4, and E8 were grouped differently from events E6 and E12 (Table 2).

Effect of polyploidy events on stomata

Different groups of acclimatized plantlets were observed after 110 days of evaluation for the epidermis of their leaves. The mean stomata size in the tetraploid events was found to be higher than in the diploid events (Table 2), where the polar and equatorial diameters formed a group for the tetraploid events E3, E4, E6 and E12, and another group for the diploid event E1 and the tetraploid event E8. Both the epidermal cells and stomata were larger in the tetraploids than in the diploid event (Figure 1).

The stomata of the tetraploid events, E3, E4, E6, E8, and E12, were more functional compared with the diploid event E1 (Table 2).

Stomata in the tetraploid events were found to be smaller than the diploids (Table 4 and Figures 1E and 1F). Also, the mean stomatal density on the abaxial surface of the leaves was found to be higher in the diploid E1 event in comparison to all the other tetraploid events, forming separate groups (Table 2). On the adaxial surface, diploid events E1 and E8, and tetraploid E12 were grouped together, separating from the other tetraploid events, E3, E4, and E6. In general, the stomatal density was higher in the abaxial surface compared to the adaxial surface (Table 2 and Figures 1E and 1F).

Effect of polyploidy events on acclimatization

Differences in survival and rooting were observed (*P* < 0.05) between diploid and polyploid cuttings. The survival of the tetraploids was found to be lower than the control, except for E3, which showed insignificant differences. All the tetraploid events had lower rooting percentages in comparison to the other diploid events (Table 2).

Discussion

Oryzalin and trifluralin were efficient in tetraploid induction in *Eucalyptus urophylla*. Although colchicine was efficient in inducing polyploidy in many plant species (Oumar et al., 2011; Xing et al., 2011; Blasco et al., 2015), tetraploids or

Table 1. Percentage of diploid (2x), tetraploid (4x) or mixoploid (2x + 4x) plants of *Eucalyptus urophylla* plants regarding polyploidy induction treatments using trifluralin and oryzalin in 5 and 10 µM, and the control (without antimitotic substances), at 60, 100, and 170 days of *in vitro* evaluation and the ploidy events generated from each treatment.

	60 days			100 days			170 days		
Ploidy	2x	4x	2x + 4x	2x	4x	2x + 4x	2x	4x	2x + 4x
Control	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
Colchicine 5 µM	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
Colchicine 10 µM	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
Trifluralin 5 µM	72.7	18.2	9.1	81.8	18.2	0.0	72.7	18.2	9.1
Trifluralin 10 µM	83.4	8.3	8.3	83.3	16.7	0.0	75.0	16.7	8.3
Oryzalin 5 µM	69.2	7.7	23.1	84.6	7.7	7.7	69.2	7.7	23.1
Oryzalin 10 µM	91.7	8.3	0.0	91.7	8.3	0.0	91.7	8.3	0.0

Treatment	Event	60 days	100 days	170 days
Control	E1	2x	2x	2x
Trifluralin 5 µM	E2	2x + 4x	2x	2x + 4x
	E3	4x	4x	4x
	E4	4x	4x	4x
Trifluralin 10 µM	E5	2x	2x	2x + 4x
	E6	4x	4x	4x
	E7	2x + 4x	4x	4x
Oryzalin 5 µM	E8	4x	4x	4x
	E9	2x + 4x	2x	2x + 4x
	E10	2x + 4x	2x + 4x	2x + 4x
	E11	2x + 4x	2x	2x + 4x
Oryzalin 10 µM	E12	4x	4x	4x

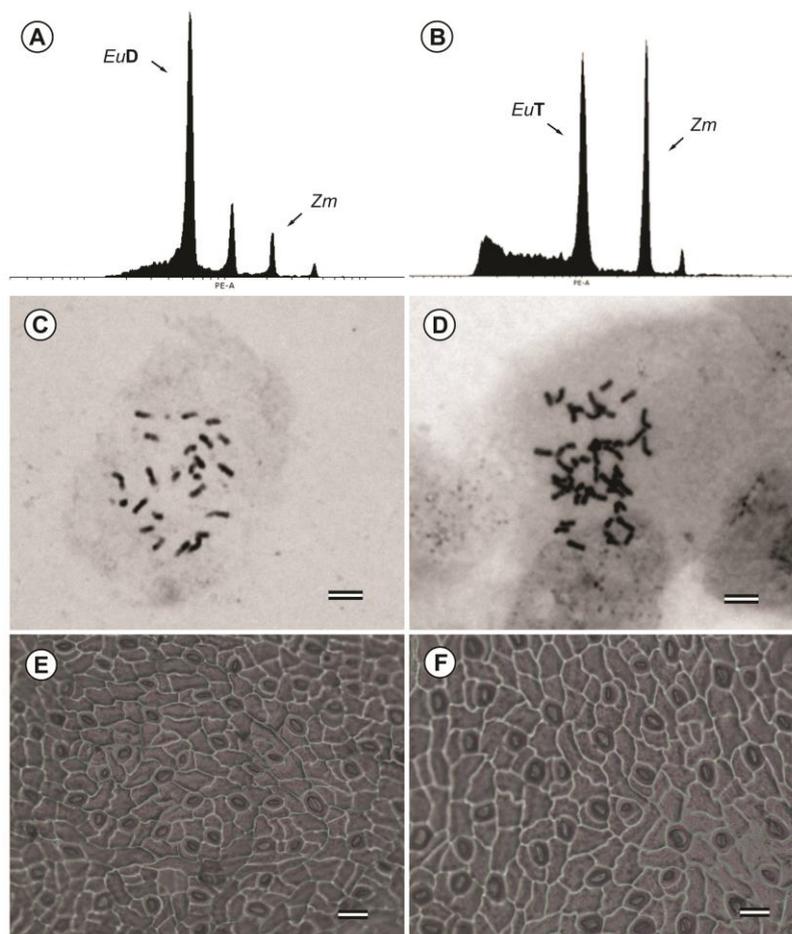**Fig 1.** Histogram of *Eucalyptus urophylla* nuclear DNA content (A) clone diploid = 1.41 pg (*Eu*D) and (B) tetraploid = 2.86 pg (*Eu*T). and *Zea mays* = 5.43 pg (*Zm*); metaphase of *Eucalyptus urophylla* mitosis (C) 2n = 24 and (D) 4n = 48; Leaf epidermal of (E) abaxial surface of diploid and (F) abaxial surface of tetraploid plants of *Eucalyptus urophylla* clone evaluated at 110 days. Bar: 50 µm

Table 2. Photosynthetic pigments, stomatal measure parameters, and survival and rooting of *Eucalyptus urophylla* diploid and polyploid events. Abbreviations: PD: polar mean diameter (µm); ED: equatorial mean diameter (µm); SF: stomatal functionality (PD/ED ratio - µm); AbSD: stomatal density of the abaxial leaf surface (mm^2); AdSD: stomatal density of the adaxial leaf surface (mm^2)

Event	Chlorophyll *a*	Chlorophyll *b*	Carotenoids	Total chlorophyll
	------------------------------ µg m^{-2} ------------------------------			
E1	33.36 b	14.49 a	7.69 a	47.85 a
E3	36.79 a	17.14 a	8.43 a	52.48 a
E4	38.75 a	15.69 a	8.29 a	55.88 a
E6	26.83 c	11.24 c	6.52 b	38.07 b
E8	33.33 b	14.74 a	7.60 a	48.07 a
E12	31.21 b	13.46 b	7.16 b	44.66 b

Event	PD (µm)	ED (µm)	SF (µm)	AbSD (mm²)	AdSD (mm²)
E1	19.86 b	14.97 b	1.33 b	193.81 a	6.62 a
E3	26.55 a	17.68 a	1.51 a	80.86 c	3.92 b
E4	27.17 a	17.84 a	1.52 a	90.90 c	1.23 b
E6	27.34 a	17.94 a	1.53 a	98.25 c	3.19 b
E8	21.38 b	14.43 b	1.49 a	145.79 b	7.11 a
E12	26.46 a	17.51 a	1.51 a	99.24 c	6.62 a

Event	Survival (%)	Rooting (%)
E1	84.44 a	75.56 a
E3	71.11 a	31.11 b
E4	31.11 b	4.44 c
E6	53.33 b	46.67 b
E8	46.67 b	40.00 b
E12	44.44 b	24.44 b

Means followed by equal letters in the same column do not differ by Scott-Knott's test at 5% probability.

even mixoploids in *Eucalyptus urophylla* did not appear to be useful for this compound.

In *Brassica napus*, polyploidization rates with oryzalin and trifluralin herbicides were observed to be higher than those obtained with colchicine, whereas, mixoploids were observed only in treatments with colchicine (Klíma et al., 2008). In *Rosa helleborus*, colchicine was seen to be ineffective for chromosome duplication, whereas tetraploids were induced with oryzalin (Dhooghe et al., 2011). In *Rosa multiflora*, trifluralin and colchicine were efficient in tetraploid induction (Feng et al., 2016). In *Hymenaea courbaril*, tetraploid induction was observed after 96 h of exposure with trifluralin (Bona et al., 2016).

The use of 5 µM trifluralin was found to be the most effective treatment in the production of stable tetraploid events. Klíma et al. (2008) also obtained stable polyploid plants of *Brassica napus* using trifluralin. Pereira et al. (2017) have shown earlier that in the progenies of tetraploid plants, the stabilization in the chromosome amount and DNA content required time. In accordance with these reports, it can be stated that the success rate depends on continuous and rigorous monitoring and selection.

To obtain polyploids, the antimitotic agent acts on the fibers of the mitotic spindle during cell division, preventing their polymerization or promoting their fragmentation, thus, not allowing chromosome separation in the anaphase (Pereira et al., 2012). The cells initiate their next cycle with twice the amount of DNA (Pereira et al., 2012). When the genetic material duplication occurs, only partly in cells of the same plant, mixoploids are formed, which may have different ploidy levels in different tissues.

The photosynthetic pigments are good indicators of the physiological status of the plant, such as chlorophyll that is mainly responsible for photosynthesis (Alvarez et al., 2012). Corrêa et al. (2016) observed greater biomass accumulation

and photosynthetic activity in tetraploid plants of *Pfaffia glomerata* than in diploids. Also, polyploid plants of *Thymus persicus* had darker green leaves than diploid, which suggests an association with higher photosynthetic pigment content (Tavan et al., 2015).

In relation to the stomatal functionality, all tetraploid events (E3, E4, E6, E8, and E12) showed similar behavior that actually differed from the diploid E1 event. The results showed larger epidermal cells and smaller stomata in tetraploid plants as compared to the diploid plants. Several other authors also found differences in the stomata size and density between different ploidy levels (Yang et al., 2006; Gomes et al., 2014; Pansuksan et al., 2014; Mansoureh et al., 2015; Shi et al., 2015; Tavam et al., 2015; Xie et al., 2015). The increase in the size of some of the organs in the polyploid plants occurs due to the fact that the cells with a higher chromosome number need to maintain a constant proportion of cytoplasmic/nucleus volume, so, organ increase is, in general, expected (Rauf et al., 2006). As the cell becomes larger, its volume increases faster than its surface area, and nutrient uptake is reduced (Kondorosi et al., 2000), leading to reduced cell division, thereby explaining the lower stomatal density in tetraploid plants. Besides, a possible explanation for this could be an increase in energy demand to support cell division in higher polyploids (Tsukaya 2008). This phenomenon has been postulated as the "high-ploidy syndrome", where higher ploidy levels exhibited enhanced cell expansion but reduced cell division (Tsukaya, 2008). In addition to shifting the production of biomass, polyploidy can also have an impact on biomass composition (Corneillie et al., 2018). Interestingly, the cell wall structure of *Arabidopsis thaliana* polyploids, most notably hexa and octaploids, showed characteristics of immature secondary cell walls, with an increased amount of extensin, arabinogalactan proteins, and

MPS and decreased levels of cellulose and lignin, along with reduced thickness (Corneillie et al., 2018). This study revealed that the basic somatic ploidy level negatively correlated with lignin and cellulose content, and positively correlated with the matrix polysaccharide content (i.e., hemicellulose and pectin) in the stem (Corneillie et al., 2018). This was shown earlier in tetraploid willow trees, which contained less lignin and were taller than their diploid counterparts (Serapiglia et al., 2015). Low rooting percentage and the consequent mortality of tetraploids in relation to the diploids can be explained by the aforementioned reduction in the cell division in these plants. Residual effects of trifluralin and oryzalin may have affected the cuttings vigor and their rooting ability. Gomes et al., (2014), studying *Pfaffia glomerata* polyploids, observed that tetraploid plants had lower root dry weight than the diploids. Nasser et al., (2010), studying *Manihot esculenta* propagation observed that rooting percentage of diploid cuttings was greater than tetraploid cuttings, but the roots of the tetraploid hybrids were larger than the diploids. These authors justify rooting difficulties as a consequence of the alterations in the anatomical structures affected by polyploidy. Regardless of few negative effects of polyploidy induction on the vegetative propagation of *Eucalyptus urophylla* through cuttings, rooting capacity can be recovered by tetraploid events over time, since cells can overcome cell division reduction (Kondorosi et al., 2000), and thus, normal nutrient input in the cell returns. Furthermore, silvicultural techniques can also be applied to clonal cultures to increase the vigor of the tetraploid plants (Xavier et al., 2013).

Materials and methods

Polyploidy induction and developmental evaluation of plants were carried out at the Tissue Culture Laboratory II of the Institute of Applied Biotechnology for Agriculture (BIOAGRO) and at the Forest Engineering Research Center, both at the Federal University of Viçosa. The ploidy evaluation was conducted at the Genetics and Biotechnology Laboratory of the Federal University of Juiz de Fora.

Plant material and polyploidy induction

The material was gathered from the multiplication stage of a micropropagated *Eucalyptus urophylla* clone, with 25 subcultures. Shoot apical meristems were removed from *in vitro* tufts and were inoculated into the test tubes containing 10 mL JADS culture medium (Correia et al., 1995) T1: [30 g L^{-1} sucrose (Vetec$^®$), 100 mg L^{-1} myo-inositol (Sigma$^®$), 800 mg L^{-1} PVP (polyvinylpyrrolidone-Vetec$^®$), 0.5 mg L^{-1} BAP (benzylaminopurine - Sigma$^®$), 0.01 mg L^{-1} NAA (Sigma$^®$) and 7 g L^{-1} agar (Merck$^®$)], and varying types and concentrations of the antimitotic substances: colchicine (Sigma$^®$), trifluralin (Gold Nortox) and oryzalin (Sigma$^®$) at the concentrations of 5 or 10 μM. One shoot apical meristem was inoculated into each test tube. The experiment was performed in a completely randomized design in a factorial scheme 3x2+1: three types of antimitotic substance, two concentrations of each and one additional control (without antimitotic substance), with 12 replicates for each treatment.

In vitro multiplication

After 15 days in polyploidy induction medium, the plantlets formed from the elongation of shoot apical meristems were transferred to a new JADS T1 medium, where they remained for 30 days. The plantlets were then transferred every 30 days to JADS T1 for further multiplication. Six subcultures were eventually performed.

The parts of the plantlets were then transferred to a 250 mL glass flask containing 40 mL of JADS T2 medium [30 g L^{-1} sucrose, 100 mg L^{-1} myoinositol, 800 mg L^{-1} PVP, 7 g L^{-1} agar, 0.05 mg L^{-1} BAP and 0.25 mg L^{-1} IBA (Sigma$^®$)] after six subcultures. Four tufts containing six to eight buds were then inoculated into each flask.

For all the *in vitro* steps, the pH of the media was adjusted to 5.8. The media was sterilized in an autoclave under the pressure of 1.5 atm and a temperature of 121 °C for 20 min. All cultures were maintained in a growth room at 25 ±2 °C, in a 16-h photoperiod and irradiance of 33 μmol m^{-2} s^{-1} (quantified by radiometer - LI-COR$^®$, LI-250A Light Meter) supplied with two tubular fluorescent lamps (Special Daylight, 40 W, Osram$^®$, Brazil).

DNA content analysis via flow cytometry was then performed after 60 and 100 days of induction (multiplication stage), and 170 days after induction (elongation stage).

Acclimatization

In vitro elongated microshoots larger than 2 cm of all polyploid treatments were transferred to a nursery for plantlet formation. At 110 days, DNA content was assessed using flow cytometry employing three replicates per event. Chromosome counts, photosynthetic pigments quantification, stomatal size, and density analysis were also performed. For these evaluations, four replicates per event were used.

DNA content analysis

Leaf fragments of 2 cm² of each plantlet were taken for the ploidy analysis. Maceration occurred in conjunction with the internal standard, *Zea mays* (2C DNA = 5.43 pg) (Arumaganathan and Earle 1991). The maceration was performed using a scalpel blade to release the nuclei into suspension in a Petri dish containing 1 mL of cold LB01 buffer (Dolezel et al., 1989). The nuclei were stained by adding 25 μg of propidium iodide (PI, Sigma$^®$, USA). The analysis was performed using the FACSCantoII^{TM} (Becton, Dickinson and Company, USA) flow cytometer, and histograms were obtained with Flowing Software 2.5.1. The 2C content of DNA (pg) was estimated according to the Eq. (1):

$$2C \text{ content of DNA (pg)} = \frac{\text{Mean of G1 peak of } E. \text{ uroplylla} \times 5.43}{\text{Mean of G1 peak of } Z. \text{ mays}} \quad (1)$$

Chromosome count

The root tips of the natural diploids were collected from the plantlets grown in the greenhouse. Root tips of ≈ 1.5 cm length were treated with 2 mM 8-hydroxyquinoline (Sigma$^®$, USA) for 285 min at 10 ºC, fixed in Carnoy solution (3:1 ethanol: glacial acetic acid), and stored in a refrigerator for 24 h. The material was then washed with distilled water and digested in an enzyme mix (cellulase 4%; pectolyase 1%; and hemicellulose 0.4%) and diluted in citrate buffer at 37 ºC for 7 h (Carvalho and Carvalho, 2016). The slides were prepared using the air-drying technique (Carvalho and Saraiva, 1993) and stained with 2% Giemsa solution (Merck$^®$, Germany). The material was further analyzed under a light microscope

(Olympus® BX51, Japan). After chromosome counting for each treatment with antimitotic agents, the replicates of these treatments were sub-classified into polyploidization events according to the diploids and polyploids (or the combination between them) produced.

Quantification of photosynthetic pigments

Two leaf discs of the second pair of leaves (6 mm diameter) were incubated in 2 mL of DMSO saturated with $CaCO_3$ (Santos et al., 2008) and kept in the dark for 48 h at room temperature. After the incubation period, the absorbance was determined in a GeneSys 10 UV/Visible spectrophotometer (Thermo Scientific, Madison, WI) using a 10 mm optical path quartz cuvette. The wavelengths and the equations for calculating the concentrations of chlorophylls *a*, *b*, total chlorophylls, and carotenoids were based on the methodology described by Wellburn (1994).

Size and stomatal density

For stomatal visualization, the second pair of leaves was used. The abaxial and adaxial surfaces were collected and glued with cyanoacrylate ester (SuperBonder®) on histological slides (Segatto et al., 2004). Images of the epidermis were collected using a photomicroscope (AX70TRF, Olympus Optical, Tokyo, Japan) equipped with the U-Photo system. The mean diameter of the stomata was calculated as the polar diameter, the equatorial diameter and the stomatal function (polar diameter ratio/equatorial diameter) (Castro et al., 2009) (*Streptococcus* spp.) and the stomatal density (number of stomata per mm^2 of leaf area) (Segatto et al., 2004) of the abaxial (AbSD) and adaxial (AdSD) surfaces of the leaves.

Rooting and survival of small cuttings

The microshoots were transferred to gutters suspended with a semi-hydroponic system in a bed of sand, forming the clonal mini garden. In order to evaluate rooting, strains of *Eucalyptus* cuttings established in the clonal garden were used. The cuttings with one to two pairs of leaves reduced by half were collected, packed in Styrofoam boxes with water, and staked in the substrate. After collection and before staking, their bases (about 2 cm) were dipped in IBA solution at 1000 mg L^{-1}. The cuttings remained in the greenhouse for 45 days, where survival and rooting percentages were evaluated. The treatments consisted of tetraploid and diploid events, in a randomized block design, with four replicates and 45 cuttings/replicate.

Statistical analysis

Data from the photosynthetic pigments, stomatal measure parameters along with survival and rooting of *Eucalyptus urophylla* diploid and polyploid events were submitted to one-way analysis of variance followed by the Scott & Knott's test (Scott and Knott, 1974) at a significance level of 5%.

Conclusion

Tetraploid plants of *Eucalyptus urophylla* were efficiently induced by oryzalin and trifluralin treatments. Trifluralin at 5 µM provided higher induction, as well as greater ploidy stability. Diploid plants had 1.41 pg of nuclear DNA and 22 chromosomes on an average, while tetraploid plants showed 2.86 pg and 44 chromosomes. Also, tetraploid plants, in comparison with diploids, showed a higher amount of chlorophyll, reduced number of larger stomata, and lower rooting and survival in clone propagation, which, however, can be mitigated through the silvicultural techniques. This work is a pioneer study in the induction of polyploids in *E. urophylla* and will generate several biotechnological possibilities for the breeding of this highly relevant forest species.

Acknowledgements

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