THAISE DA SILVA SOUZA

POTENTIAL FOR USE OF POLYPLOIDY IN EUCALYPTUS BREEDING PROGRAMS

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento de Plantas, área de concentração em Genética e Melhoramento de Plantas, para a obtenção do título de Doutor.

Profa. Dra.Vânia Helena Techio
Orientadora

Prof. Dr. Magno Antonio Patto Ramalho
Coorientador

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POTENCIAL PARA O USO DA POLIPLOIDIA EM PROGRAMAS DE MELHORAMENTO DE EUCALIPTO

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Dra. Fabiana Rezende Muniz	Suzano SA
Dr. Aurélio Mendes Aguiar	Suzano SA
Prof. Dra. Giovana Augusta Torres	UFLA
Prof. Dr. Magno Antonio Patto Ramalho	UFLA

Profa. Dra.Vânia Helena Techio
Orientadora

Prof. Dr. Magno Antonio Patto Ramalho
Coorientador

LAVRAS – MG
2021

To the progress of women in science.

OFEREÇO

To my Mom, Dinomá, a powerful woman!

DEDICO

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"Whatever you are, be a good one."

Abraham Lincoln

ABSTRACT

The induction of polyploidy in *Eucalyptus* can be one of the alternatives to boost eucalypt breeding since additional gains through conventional plant breeding may not meet new production demands. After artificial polyploidization, it is necessary to certify the effectiveness of the duplication via cytogenetic analyses, including chromosomal counts and nuclear DNA quantification. Another alternative is to indirectly evaluate the chromosome duplication by detecting changes in anatomical and morphological traits, such as stomata and leaf area measures, and wood anatomy studies. Besides, the confirmation of the clone performance in field conditions is essential to validate the importance of this technique. This study aimed: i) to certify the chromosome duplication in artificially polyploidized clones, using cytogenetic analysis, flow cytometry, and leaf anatomy measurements; ii) to compare the performance of *Eucalyptus* diploid and polyploid clones with the same genetic origin by analyzing the physical and anatomical properties of the wood to provide more information on wood quality for pulp and paper production; and iii) to evaluate the performance of these clones under field conditions to verify if they are more adapted and stable for forest exploration, and if the performance of polyploid depends on its diploid of origin. Clones of *Eucalyptus grandis x Eucalyptus urophylla* diploids and artificial polyploids produced by Suzano SA were evaluated. Chromosomal counts and DNA content estimation allowed the identification of artificially polyploidized clones and mixoploids. Polyploid clones presented larger stomata diameter than diploids and leaf area significantly increased as a function of the ploidy level. Wood samples from these hybrids were analyzed and the results suggested that polyploid eucalypt clones presented lower basic density and fibers with higher length and wall thickness than diploids, which improves fiber accommodation in the paper formation and increases its strength, indicating the potential use of polyploids in eucalypt pulp and paper production. Measurements of DBH (diameter at breast height) and MAI (mean annual increment) of three-year-old trees were evaluated and the analyzes allowed to verify that polyploids differed in adaptability and stability, and in most cases, they exhibit lower performances than diploids. The relative behavior of polyploid clones does not depend on the performance of the diploid that originated it since the most adapted polyploid clones were originated from the diploid clone with the lowest performance. These results indicate that the potential for use of polyploids in eucalypt breeding is promising, although there is a need for more studies to answer the questions that remain.

Keywords: *Eucalyptus* breeding, polyploidy, wood quality, cytogenetics, quantitative genetics.

RESUMO

A indução de poliploidia é uma alternativa para impulsionar o melhoramento do eucalipto, uma vez que a obtenção de ganhos adicionais por meio do melhoramento convencional pode não fornecer o incremento na produtividade necessário para atender às novas demandas. Após a poliploidização artificial, é fundamental a certificação da efetividade do processo por meio de análises citogenéticas que incluam contagens cromossômicas e quantificação do DNA nuclear. Outra alternativa é a avaliação indireta de características anatômicas e morfológicas, tais como análises estomáticas e mensurações da área foliar, e estudos de anatomia da madeira. Além disso, a confirmação do desempenho do clone em condições de cultivo é essencial para validar a importância desta técnica. Este estudo teve por objetivo: i) certificar a duplicação cromossômica de clones de eucalipto poliploidizados artificialmente empregando análises citogenéticas, citometria de fluxo e anatomia foliar; ii) comparar o desempenho de clones diploides e poliploides de eucalipto com mesma origem genética por meio de análises de propriedades físicas e anatômicas da madeira com o intuito de obter informações da qualidade da madeira para a produção de polpa e papel; e iii) avaliar o desempenho desses clones sob condições de cultivo para verificar se eles são mais adaptados e estáveis para a exploração florestal, e se o desempenho do poliploide depende do seu diploide de origem. Clones de *Eucalyptus grandis x Eucalyptus urophylla* diploides e poliploides artificiais obtidos pela Suzano SA foram avaliados. As contagens cromossômicas e a quantificação de DNA permitiram identificar clones poliploidizados artificialmente e mixoploides. Os clones poliploides apresentaram maior diâmetro estomático que os diploides e a área foliar aumentou significativamente em função do nível de ploidia. Amostras de madeira desses híbridos foram analisadas e os resultados sugeriram que clones poliploides apresentaram menor densidade básica e maiores comprimento e espessura da parede das fibras quando comparados com os diploides, o que melhora a acomodação das fibras na formação do papel e aumenta a sua resistência, indicando o potencial do emprego de poliploides na produção de polpa e papel em eucalipto. Mensurações de DAP (diâmetro a altura do peito) e IMA (incremento médio anual) das árvores aos três anos foram avaliadas e as análises permitiram verificar que os poliploides diferiram em adaptabilidade e estabilidade, na maioria dos casos, com desempenho inferior aos diploides. O comportamento relativo dos clones poliploides independe do desempenho do diploide que o originou, uma vez que os clones poliploides mais adaptados foram originados do clone diploide de menor desempenho. Esses resultados indicam que o potencial para uso da poliploidia no melhoramento de eucalipto é promissor, contudo, existe a necessidade de mais estudos a respeito do tema para responder questionamentos que ainda permanecem.

Palavras-chave: melhoramento de eucalipto, poliploidia, qualidade da madeira, citogenética, genética quantitativa.

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FIRST PART

1 INTRODUCTION

The *Eucalyptus* is the forest genus most widely planted and studied in the world (MORA; GARCIA, 2000; SILVA, 20005; FAO, 2006; MYBURG et al., 2014; CASTRO et al., MENGISTU et al., 2020) and has been preferentially used in Brazilian forestry as a result of good adaptation to different soil and climate conditions, high yield, and multiple uses of its wood (SOUZA et al., 2017). The area of *Eucalyptus* plantations in Brazil reaches 6.93 million hectares, which represents 77% of the total planted forests in the country, and 46.5% of the wood production from these reforestations is destined for the production of pulp, paper, and paper manufacturers (IBÁ, 2020).

Although the success with eucalypt breeding in Brazil has been enormous in recent years (RAMALHO et al., 2021), the growing demand for wood, mainly for pulp production, demands that productivity be even higher. There are some alternatives to increase productivity, one of which is to improve management practices, and the other option is to obtain new clones with better performance than the existing ones.

However, as the productivity level of the clones is already high, conventional plant breeding may not provide the yield increase needed to meet new demands. Thus, other alternatives have been sought to accelerate the process, such as speed breeding technologies (WATSON et al., 2018), the use of molecular markers linked to genomic selection (BALLESTA et al., 2020; MOURA et al., 2019; MULLER et al., 2017; GRATTAPAGLIA, RESENDE, 2011), the application of the clonal progeny test (RESENDE, 2002), and the induction of artificial polyploidization (CASTILLO et al., 2020; FERNANDO et al., 2019; HAN et al., 2011).

In recent years, companies have been encouraged to obtain polyploids to achieve a relatively fast and expressive volumetric increment in wood (CASTILLO et al., 2020). In eucalypt studies, some reports were found regarding the use of polyploidy induction methodologies (KAMPOOR; SHARMA, 1985; MARITZ, 2008; HAN et al., 2009; LIN et al., 2010; HAN et al., 2011; FERNANDO et al., 2019; CASTILLO et al., 2020). Nevertheless, no reports were found on the performance of the plants obtained.

In general, the most widely known consequence of polyploidization is the *gigas* effect, which refers to the enlargement of plant organs due to the increased number of gene copies (SATTLER et al., 2016). In this way, will polyploids, due to having a greater number of alleles

copies, effectively contribute to increasing the wood volume? Similarly, does the higher number of copies further mitigate the effect of the clone by environment interaction? What would be the possible effects of polyploidy on wood anatomy? Is there an ideal ploidy level among the cultivated trees? Do mixoploids occur? Is there variation between polyploid clones obtained from a diploid clone? If so, can this variation be used to improve the selection process?

Many questions remain and more studies are needed to identify the best strategy for continuing research aimed at the use of polyploidy in eucalypt breeding in Brazil. In this context, this study aimed to evaluate the potential for use of polyploid *Eucalyptus* clones in breeding programs in the country. Specifically, the analyzes were performed to compare the performances of eucalypt diploid and polyploid clones with the same genetic origin by analyzing the physical and anatomical properties of the wood to extend the information on wood quality for pulp and paper production; to certify the chromosome duplication in artificially polyploidized eucalypt clones using cytogenetic analysis, flow cytometry, and leaf anatomy measurements; and to evaluate the performance of these clones under field conditions to verify if they are more adapted and stable for forest exploration and if the performance of polyploid depends on its diploid of origin.

2 LITERATURE REVIEW

2.1 *Eucalyptus* forestry in Brazil

Eucalyptus is the forest genus most widely planted and studied in the world (MORA; GARCIA, 2000; SILVA, 20005; FAO, 2006; MYBURG et al., 2014; CASTRO et al., MENGISTU et al., 2020). Throughout their natural evolution, plants of the genus have developed efficient adaptive mechanisms to grow quickly under unfavorable conditions and water deficit, temperature and nutritional stresses, among others, which explains the large number of species in nature and their wide dispersion in the regions of origin (PALUDZYSZYN FILHO et al., 2006).

Eucalypt has been preferentially used in Brazilian forestry due to its characteristics of adaptation to the most diverse climate and soil conditions, fast growth, and the multiple use of wood (SOUZA et al., 2017). Among the main species used, *Eucalyptus grandis* and interspecific hybrids, mainly with *Eucalyptus urophylla* species, continue to be the most important, due to the intensive and growing use in industry (PINTO JÚNIOR; GARLIPP, 2008).

In Brazil, eucalypt found excellent climate and soil conditions to develop, with higher productivity than other producing countries (BRACELPA, 2011). The planting-to-harvest cycles of species in the country is five to seven years, lower than in countries such as South Africa (8-10 years), Chile (10-12 years), Portugal (12-15 years) and Spain (12-15 years) (VOTORANTIM CELULOSE E PAPEL, 2004; IBÁ, 2020).

The wood produced is destined for pulp and paper, energy, steel industry, panels and laminate flooring, wood charcoal, solid products, lumber, and plywood (PALUDZYSZYN FILHO; SANTOS, 2011, IBÁ, 2020). Investments in research and development, new uses and innovation in products and processes are extremely important to the forest sector, from raw materials to the final product and waste management. For example, products manufactured with cellulose range from traditional products like paper to thickeners used in ice cream (industrial as well as artisanal), makeup and cosmetics, personal hygiene products like toothpaste, pharmaceuticals, and tissues. One market that has grown significantly in recent years is the textile industry (nearly 7% of the textile market comes from soluble cellulose) (IBÁ, 2020).

In Brazil, the total area of planted trees totaled 9 million hectares in 2019 (IBÁ, 2020). Of this total, the majority (77%) represents eucalypt trees, with 6.97 million hectares, and are located mainly in the States of Minas Gerais (28%), São Paulo (17%) and Mato Grosso do Sul (16%) (IBÁ, 2020). The greater concentration of forest plantations in the Southeast region of

the country is justified by the location of the main forest industries (SOUZA et al., 2017). However, there is an expansion trend of plantations to other regions of the country.

Of the total 9 million hectares of trees planted in Brazil, the paper and pulp segment concentrates 34% of this area, followed by the segments of independent producers (29%), steel and charcoal (14%), financial investors (10%), wood panels and laminate flooring (6%), lamber, plywood and other solid products (4%), and others (3%) (IBÁ, 2017). Most of the area with eucalypt plantations, around 70%, is destined to the pulp and paper segment, followed by the steel and charcoal, and wood panel segments (SOUZA et al., 2017). Brazil continues to be a global reference in the production of cellulose pulp. In 2019, the country maintained its position as the second-largest producer, reaching 19.7 million tons of production, and the largest exporter, as 75% of all production was exported, totaling 14.7 million tons (IBÁ, 2020).

The main competitive advantage of Brazil, in addition to favorable environmental conditions, is its forestry technology, based on programs for genetic tree improvement and clonal multiplication of eucalypt developed in the last fifty years (ALFENAS et al., 2004; RAMALHO et al., 2021). However, plant breeding is a time-consuming step and may not provide the required increase in productivity to meet new demands in a short time. Therefore, it is necessary to use other tools to turn the clonal evaluation process as effective as possible (SOUZA et al., 2017).

Although many clones are highly productive in wood and pulp, they still present excessive lignin content (between 22% and 30%), extractive contents, physical-mechanical and anatomical characteristics, basic wood density, among others (SILVA, 2016). These aspects influence growth tensions and reflect on wood processing, and its use as a raw material (PALUDZYSZYN FILHO; SANTOS, 2011). In this sense, the use of biotechnological tools such as the induction of synthetic polyploids enables new studies and enrichment of eucalypt breeding programs to meet new demands technologies (GRATTAPAGLIA; KIRST, 2008; MARITZ, 2008).

2.2 *Eucalyptus* breeding in Brazil

The first studies on the cultivation of eucalypt in Brazil began in 1904, with Edmundo Navarro de Andrade at the Companhia Paulista de Estradas de Ferro, who introduced several species and provenances from Australia, noting good adaptation of *Eucalyptus* in the country. Thus, the planting was encouraged as it provided, in less time, fuel and wood for the railroad (COUTO; MÜELLER, 2008; VENCOVSKY; RAMALHO, 2000; FERREIRA; SANTOS,

1997). It is noteworthy that the first introduction of *Eucalyptus* species was carried out in 1825 in the Botanical Garden of Rio de Janeiro (SILVA; BARRICHELO, 2006). However, breeding truly began some years later, in 1941, at Instituto Agronômico de Campinas (IAC) with Carlos Arnaldo Cruz (CASTRO et al., 2016). Nevertheless, it was only from the 1960s onwards, at the initiative of some private companies and also the public sector, that the breeding began to be performed systematically (SILVA; BARRICHELO, 2006).

In 1967, the Aracruz Celulose company, now called Suzano, began planting eucalypt in Espírito Santo State. Quickly verified that the species with the best wood productivity was *E. grandis*. However, they verified that in this species there was a large proportion of individuals susceptible to canker (disease caused by the fungus *Cryphonectria cubensis*). For this reason, the company also started working with *E. urophylla*, which associated high canker resistance and good wood yield (BISON, 2004). Soon they also perceived that hybrids of *E. grandis* and *E. urophylla* exhibited considerable heterosis in terms of wood volume (RAMALHO et al., 2021).

The great genetic advance occurred when Aracruz researchers glimpsed the possibility of carrying out clonal planting. They then started to select superior trees, especially in commercial plantations, the majority, natural hybrids involving *E. grandis* and *E. urophylla*. The first commercial clonal eucalypt plantation in Brazil was established in 1979, about 12 years after the company began its cultivation. For this, the vegetative propagation of meristematic cells was used (FERREIRA; SANTOS, 1997).

The eucalypt plantation in Brazil is predominantly formed by clones with high mean productivity, reaching yields in the order of 45-60 m^3 ha^{-1} $year^{-1}$ (SILVA, 2008). About 70% of the planted area comes from clones (PALUDZYSZYN FILHO; SANTOS, 2011). Since the introduction of cloning, Brazilian eucalypt plantations has undergone great advances, especially with regard to seedling production techniques, use of superior clones, disease control, soil preparation and cultural treatments necessary for the establishment of high productivity forests (ALFENAS et al., 2004). 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).

From the 1980s on, molecular markers began to be used in eucalypt, as in other cultivated species, in practically all companies (RAMALHO et al., 2021). After DNA sequencing, various studies, especially using simulation, showed the potential for use of genome-wide selection, above all to accelerate the selection process (RESENDE et al. 2008). In addition, due to the need to accelerate the process from hybridization up to recommendation

of the clone, some companies of the sector have begun what is called the clonal progeny test. Theoretically, this breeding process is highly efficient (RESENDE; HIGA, 1994) because the clones are evaluated without the initial progeny test. Specifically, the seeds obtained in the recombination lot, i.e., seeds of the half-sib or full-sib progenies, are cloned in the nursery soon after germination. The clones of the different individuals of each progeny are evaluated and, thus, their performance depends on the performance of the progeny that gave rise to it. The assumption is that the clone is better evaluated and that it is not necessary to perform the progeny test before the clonal test (RAMALHO et al., 2021).

2.3 Taxonomy and cytogenetics of *Eucalyptus*

The genus *Eucalyptus* L'Hér belongs to the Myrtaceae family and is the most widely cultivated woody angiosperm species in the world (LADIGES et al., 2003). *Eucalyptus* is predominantly native to Australia and nearby regions, where they occur in different habitats (RIBEIRO et al., 2016). Plants of this genus are highly diversified and exhibit significant adaptability and phenotypic plasticity (RIBEIRO et al., 2016). They can be giants like *E. regnans* or shrubs like *E. pulverulenta* (GRATTAPAGLIA et al., 2012). A taxonomic review (BROOKER, 2000) of eucalypt trees recognizes just over 700 species belonging to 13 subgenera, including *Corymbia* K.D.Hill & L.A.S., *Johnson* (bloodwood eucalyptus) (HILL; JOHNSON, 1995), and *Angophora* Cav. (LADIGES, 1997). Most species belong to the subgenus *Symphyomyrtus* Schauer, which is divided into 14 sections (BROOKER, 2000). Of all these sections, only three are used in forestry for industrial purposes: section *Latoangulatae* (*E. grandis* and *E. urophylla*); *Exsertaria* section (*E. camaldulensis*), which grows in tropical and subtropical regions; and section *Maidenaria* (*E. globulus*), which grows in temperate regions (GRATTAPAGLIA et al., 2012). These species and their hybrids are among the main sources of biomass in the world, and are the main raw material used in pulp and wood production (RIBEIRO et al., 2016).

All this diversity among *Eucalyptus* species, related to its economic importance, requires more studies that expand the knowledge about its characteristics, allowing the development of advanced improvement strategies for species of the genus.

Cytogenetic studies on eucalypt can provide valuable information about the system and evolution of the group (MATSUMOTO et al., 2000). The determination of the chromosome number presents an important role in the characterization of species and can be useful to study phylogenetic relationships between and within groups (SENN, 1938).

The chromosome number and morphology of the genus *Eucalyptus* have been investigated in several studied. Ruggeri (1960) evaluated 62 species and all showed 2n = 22 chromosomes, except *E. reduncea*, *E. corynocalyx*, and *E. flocktoniae*, which apparently were aneuploid with 2n = 24. Other authors confirmed 2n=22 chromosomes for *Eucalyptus* species, such as the descriptions by Haque (1984) for *E. torelliana, E. citriodora, E. camaldulensis* and *E. tereticornis* and by Matsumoto et al. (2000) for E. *deanei, E. dunni, E. grandis, E. maculata, E. propinqua, E. saligna* and *E. tereticornis*. Later, Bachir and Abdellah (2006) corroborated this number of chromosomes in 54 species, but described four species with 2n = 20, 24 and 28 chromosomes. These results showed the constancy and homogeneity in the *Eucalyptus* chromosome set, also confirmed by the karyotypic symmetry described by Matsumoto et al. (2020). This homogeneity in chromosome morphology also shows why so many hybrids of *Eucalyptus* species occur in nature and why relatively fertile F_1 hybrids are easily produced by artificial crossing between taxonomically related species (HAQUE, 1984).

The karyotypic constancy of 2n = 22 for *Eucalyptus* has already been described in 135 species (BACHIR; ABDELLAH, 2006) and the evolution process of the genus probably occurred due to structural changes (eliminations, duplications, additions and translocations) and to aneuploidies (MATSUMOTO et al., 2000). It is likely that chromosome counts based mainly on conventional cytogenetic analyzes and morphological comparisons between chromosomes may be responsible for descriptions of aneuploidies and descriptions of species with 2n = 24 (MATSUMOTO et al., 2000; OUDJEHIH and ABDELLAH, 2006) .

Myburg et al. (2014), when analyzing the genome of *E. grandis*, suggested that the genome of the genus was shaped by chromosomal duplication events and by the high rate of gene duplication in tandem. The availability of *E.grandis* genome sequence together with increasingly powerful molecular technologies provide exceptional opportunities for whole-genome comparative and evolutionary studies across species of *Eucalyptus* (RIBEIRO et al., 2016). Although there is a huge amount of molecular resources for comparative *Eucalyptus* studies, including genetic linkage maps constructed with transferable microsatellites (GRATTAPAGLIA et al., 2015), QTL mapping (GION et al., 2015), and microarray-based Diversity Array Technology (DArT) (GRATTAPAGLIA et al., 2011; STEANE et al., 2011), the knowledge of these genomes at the cytogenomic level is very scarce (RIBEIRO et al., 2016).

Techniques such as fluorescence *in situ* hybridization (FISH) are important molecular cytogenetic tools to reveal phylogenetic relationships among trees (RIBEIRO et al., 2008). It can avoid mistaken chromosome counts and even elucidate pairs in the chromosome sets (RIBEIRO et al., 2016). These last authors characterized and compared the karyotypes of six

eucalypt species (*E. grandis*, *E. globulus, E. calmadulensis*, *E. pulverulenta*, *E. cornuta,* and *E. occidentalis*) using FISH mapping with rDNA markers, which was essential for unequivocal establishment of chromosome pairs in their study.

The first study on the quantification of nuclear DNA in *Eucalyptus* by flow cytometry (MARIE; BROWN, 1993) disclosed the value of 1.13 picograms (pg) of DNA/2C and 40.1% of GC bases for *E. globulus*. Grattapaglia and Bradshaw (1994) analyzed 19 different eucalypt plants, among commercially important species and interspecific hybrids, and obtained results in pg of DNA/2C ranging from 0.77 for *C. citriodora* to 1.47 for *E. saligna*, and *E. globulus* had 1.09 pg of DNA/2C.

In another study (PRAÇA et al., 2009), flow cytometry did not reveal the occurrence of variation in the amount of DNA for *E. globulus*, *E. grandis,* and *E. urophylla* (1.05; 1.09; 1.01 pg of DNA/2C, respectively). According to the authors, this information is necessary to understand the nature and occurrence of reproductive barriers, and the genetic basis of the hybrids superiority in this genus. The effectiveness in chromosomal duplication of diploid plants of *Eucalyptus* can make it possible to obtain polyploid varieties (HAN et al., 2011), as a valuable tool in plant breeding (ALMEIDA et al., 2013).

2.4 Polyploidy in forest species

Due to its importance, some breeding programs include polyploidization as one of their strategies (PEREIRA et al., 2012). Among several advantages are the possibility of increasing the number of alleles at a locus, restoring the fertility of interspecific hybrids, obtaining lineages in a short period of time and enabling crosses when the genotypes of interest have different ploidies (BARBOSA et al., 2007; CAMPOS et. al., 2009; ISHIGAKI et al., 2009; SOUZA-KANESHIMA, 2010).

Polyploid can occur naturally by duplication of the genome in the same plant, or by crossing distinct polyploid species, being classified as autopolyploid and allopolyploid, respectively (HESLOP-HARRISON; SCHWARZACHER, 2011). Another way is the spontaneous crossing by the fusion of unreduced gametes, which can originate bilateral polyploids (by the union of two unreduced gametes) or unilateral polyploids (formed from the fusion of a reduced gamete with another unreduced gamete) (SATTLER et al., 2016).

Polyploids can also be induced through the use of antimitotic substances, which act on the spindle fibers during cell division, preventing their polymerization or promoting their fragmentation, and thus do not allow the separation of chromosomes in anaphase.

Consequently, cells start the next cell cycle with the amount of DNA duplicated (PEREIRA et al., 2012). Colchicine is the most used substance for inducing polyploidy in plants (CASTILLO et al., 2020), but physical processes (thermal shock (RANDOLPH, 1932)), biological methods, through the use of *in vitro* culture with generation of calluses and emergence of variant cells (PHILLIPS et al., 1994), and chemically or mechanically fused protoplasts can also be used (SLATER et al., 2003).

In general, the most widely known consequence of polyploidization is the *gigas* effect, which refers to the enlargement of plant organs due to the increased number of gene copies (SATTLER et al., 2016). Autopolyploids, such as those generated by induction of chromosome duplication, generally show reduced fertility due to the formation of multivalents and meiotic irregularities (STEBBINS, 1971), but may be more resistant to biotic and abiotic factors, such as temperature, drought, salinity and even resistance to pests and diseases (LEVIN, 2002).

The polyploidy induction strategy has been studied in forest species. In conifers, for example, commercial species such as *Pinus silvestres, Pinus contortia, Picea abis,* and *Larix sibirica* were polyploidized using colchicine at different concentrations (SILVA, 2016). The induction of tetraploids in *Acacia* sp. has also been studied with the purpose of incorporating polyploids in breeding programs, in order to obtain plants with low fertility and sterile when crossed with diploid individuals (SILVA, 2016).

Specifically in eucalypt, Maritz (2008) evaluated the induction potential in seeds of *E. urophylla* and *E. grandis,* and axillary buds of *E. grandis*, hybrids *E. grandis* x *E. urophylla,* and *E. grandis* x *E. nitens*, highlighting phenotypic differences after treatment with different concentrations of colchicine (0.00, 0.01, 0.03, 0.05% for 18 and 24 hours for seeds and 0.0, 0.5, 1.0, 1.5% for three consecutive days for axillary buds). Of the 17 polyploid plants of *E. urophylla* evaluated, six were tetraploids and 11 were mixoploids. In five plants of *E. grandis* polyploids obtained by seed induction, one was tetraploid and four mixoploids. As for the hybrids, only four of the 12 plants evaluated presented mixoploidy, and in *E. grandis* polyploid obtained by induction in the axillary bud, three of the six individuals evaluated were mixoploid. In this same study, the induction of polyploidy in seeds was more efficient than in axillary buds.

In another study, Lin et al. (2010) reported that concentrations of around 0.5% of colchicine for four days in axillary buds of *E. globulus* generate higher rates of polyploids, although mortality is a limiting factor (about 60% of explants), hindering the efficiency of the process. In this study there are no reports of mixoploid plants. Han et al. (2011) developed an equation to predict the maximum induction and concentration to be used in *E. grandis*, considering a series of factors that can influence the polyploidization process. The maximum

induction rate was obtained with 0.21% colchicine for 26.2 hours in axillary buds. These authors also considered that despite the prediction reaching up to 51% in the induction rate, mixoploid individuals still remain as obstacles during the entire process and maintenance of cultures. Moura et al. (2020) obtained tetraploid and mixoploid plants from *E. urophylla* clones using trifluralin and oryzalin as antimitotic agents. Castillo et al. (2020) also obtained tetraploid and mixoploid plants from *E. dunnii* clones using colchicine as a polyploidizing agent.

The main methods for evaluating polyploidization are chromosomal counts, estimation of DNA content and evaluation of morphological and anatomical characteristics (cell size, pollen grain diameter, stomata size and density). Chromosome counts are a reliable method as it determines the exact number of chromosomes in individual cells. However, it is laborious and time-consuming, allowing the assessment of a limited number of cells, being disadvantageous when it comes to analyzing a large number of plants (PEREIRA et al., 2012). To verify the ploidy level on a large scale, the analysis of nuclear DNA content has been performed using the flow cytometry technique (DOLEZEL, 1997). This is the method most frequently used to detect polyploidy, followed by chromosomal counts and the combination of both (PEREIRA et al., 2012). The advantage over chromosome counts is to allow the assessment of the ploidy level of a large number of plants, from different types of tissues and to analyze plants in the initial phase of the chromosome duplication process, since it is a non-destructive method that uses small amounts of young tissue for analysis (PEREIRA et al., 2012). Nevertheless, DNA quantification by flow cytometry provides results from a group of cells, which in some cases may lead to doubts in the results, requiring confirmation of the ploidy level by other techniques, such as chromosome counts (SCHIFINO-WITTMANN, 2001). For the analysis to be reliable, a careful standardization of techniques is necessary. Otherwise, the results may show values that do not correspond to the true amounts of DNA in the analyzed sample (MAGALHÃES, 2014).

The observation of morphological and anatomical characteristics occurs through the verification of the *gigas* effect, in which the plant organs and cells increase in volume proportionally to the increase in the amount of DNA (PEREIRA et al., 2012). However, they can also be associated with mutagenic effects caused by the polyploidizing agent used, as well as somaclonal variations (TSAI et al., 2013; REGALADO et al., 2015). The combination of these methods allows certifying the efficiency of chromosome duplication, considering that polyploidized plants are not always stable genetic and phenotypically.

In several studies on induced polyploidy, the regeneration of mixoploid plants has been reported as the main problem (PEREIRA et al., 2017). Thus, genetic and cytogenetic

monitoring of polyploidized genotypes is necessary for a better understanding of the phenomenon and to guarantee the commercial use of the individuals obtained by this process.

2.5 Genotype by environment interaction

The genotype by environment interaction (G x E) can be understood as the non-coincident behavior of genotypes in different environments and represents the main challenge for breeders. In forestry, where activities are conducted on thousands of hectares, often located in different states and with wide variation in environmental conditions, the interaction is even more challenging (SOUZA et al., 2017).

The G x E interaction has an enormous importance in plant breeding because most characters of economic interest are quantitative, and suffer a marked influence of this interaction (RAMALHO et al., 2012). Therefore, small variations in the environment are enough to cause significant phenotypic changes, such as the volumetric production of wood in forest species (PATIÑO VALERA; KAGEYAMA, 1988).

To minimize the effects of the G x E interaction in the selection of genotypes, there are at least three possible options: carry out ecological zoning, identify specific genotypes for each environment, and identify genotypes with greater stability (CRUZ et al., 2004). In the case of zoning, ecologically similar environments are grouped into sub-regions within which the interaction is not significant. This grouping, however, is only possible based on macro-environmental differences (years, locations, regions) making the zoning vulnerable to unpredictable variations that may occur in the environment (RAMALHO et al., 2012).

When a clone is recommended, it is expected that the environmental conditions under which it is grown during the selection process will be repeated in the future. Nevertheless, past experience shows that this does not always occur. As a result of this interaction, frustrations in recommendations of new clones are not uncommon and, evidently, there are large losses for companies (RAMALHO et al., 2021). To reduce uncertainties and make more sustainable recommendations of clones, the use of clonal composites instead of monoclonal plantings has been proposed. The idea is that the clonal composite, for example, a composite with ten clones, can function as a guarantee. That is, if one or more clones are not well adapted to the new growing conditions, the other clones can compensate the losses in the field area. In monoclonal plantings, such losses cannot be compensated (REZENDE et al. 2019).

Studies on G x E interaction in *Eucalyptus* species are abundant in Brazil (SANTOS, 2012; NUNES et al., 2002; SOUZA et al., 1993; SOUZA, et al., 2017). Most of these works

quantify the interaction and try to classify the species/provenances/clones according to their adaptability and stability (SOUZA, et al., 2017). However, studies that aim to estimate the interaction and relate it to the effect of polyploidization on the differential performance of genotypes were not found.

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SECOND PART – ARTICLES

ARTICLE 1 – CERTIFICATION OF ARTIFICIAL POLYPLOIDIZATION IN EUCALYPTUS CLONES

Article prepared according to the rules of the Tree Genetics and Genomes Journal (preliminary version)

CERTIFICATION OF ARTIFICIAL POLYPLOIDIZATION IN EUCALYPTUS CLONES

Thaise da Silva Souza, Jamile Paiva Macedo, Marco Tulio Mendes Ferreira, Isabella de Campos Moraes, Evaristo Mauro de Castro, Magno Antonio Patto Ramalho, Fabiana Bombonato Mingossi, Alexandre Alves Missiaggia, Vânia Helena Techio

ABSTRACT

The induction of polyploidy in *Eucalyptus* can lead to more productive and adapted clones with better wood quality. Such process can increase yield gains in eucalypt breeding programs, considering that conventional plant breeding may not meet new production demands. After artificial polyploidization, it is necessary to certify the effectiveness of the duplication via cytogenetic analyses, including chromosomal counts and nuclear DNA quantification. It is also possible to assess anatomical and morphological traits, such as stomata and leaf area measurements. Thus, this work aimed to certify the chromosome duplication in artificially polyploidized clones of *Eucalyptus grandis x 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 presented larger stomata diameter than diploids, and leaf area significantly increased as a function of the ploidy level. Our findings indicate that the use of polyploidy in *Eucalyptus* breeding is promising, allowing the discrimination of diploids, tetraploids, and mixoploids plants.

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

INTRODUCTION

The growing demand for wood from *Eucalyptus* L'Hér, especially for pulp production, requires more yield increases, despite the success already achieved in the last 50 years of breeding (Ramalho et al., 2021). One of the alternatives to achieve more gains is improving silvicultural and crop management practices, which could lead to the production of new clones with better performances.

However, as productivity levels of eucalypt clones are already high, conventional plant breeding may not provide the necessary increases to meet new demands. Therefore, different strategies are being employed to accelerate the process, such as genomic selection, gene editing, and polyploidization of genotypes of interest (Han et al., 2011). More recently, promising results have intensified the research on the use of synthetic eucalypt polyploids in Brazil (Souza et al., 2021).

Induced polyploidy has been pointed as a valuable tool for the genetic improvement of economically relevant plants (Almeida et al., 2013), as polyploid plants commonly display greater vigor and larger sizes (Sattler et al., 2016). Besides increasing productivity by exploiting the *gigas* effect, the main advantage of polyploidization in the *Eucalyptus* breeding is to produce triploid sterile plants, which may exhibit more vegetative growth by redirecting resources from the reproductive organs. *Eucalyptus* triploid plants, for example, have been used to prevent gene introgression due to contamination of pollen from transgenic plants (Liu et al., 2013).

After artificial polyploidization, it is necessary to confirm the effectiveness of the process through cytogenetic analyses, such as chromosome counts and estimation of nuclear DNA. For large-scale analyses, flow cytometry is a fast and easy method for estimating DNA content (Pereira et al., 2012). Nevertheless, flow cytometry results are based on a sample of cells and, in some cases, it can display ambiguous outputs regarding the ploidy level, requiring confirmation by chromosome counts (Schifino-Wittmann, 2001; 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 the chromosome duplication, by detecting changes in anatomical and morphological traits, such as stomata and leaf area measures. This method is based on the observation of increases in vigor and cell and organ sizes in polyploid plants compared to diploids, known as *gigas* effect (Sattler et al., 2016; Del Pozzo et al., 2015).

Such phenomenon was observed in *Eucalyptus polybractea* R. T. Baker plants polyploidized with colchicine (Fernando et al., 2019), in which tetraploids displayed greater diameter and lower density of stomata when compared to diploids.

In this context, the present study aimed to certify the chromosome duplication of artificially polyploidized clones of *Eucalyptus grandis* Hill ex. Maiden *x 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 forestry company Suzano SA, were evaluated considering 10 plants of each clone, five probable tetraploids (A, B, C, E, and F), and one diploid, totaling 60 plants. Tetraploid clones were obtained according to the chromosome duplication protocol used by the company.

Chromosome preparations for obtaining C-metaphases

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% pectoliase Sigma-Aldrich ®) for 2 h 45 min, at 37 ºC. Slides were prepared using the cell dissociation technique (Carvalho; Saraiva, 1993) and air-dried. The chromosome preparations were stained with the fluorochrome DAPI (4',6-diamidino-2-fenilindole) and mounted with Vectashield medium. Chromosome counts were performed on 30 metaphases per clone.

Double staining of chromosomes with CMA-DAPI

Slides were aged for seven days and subjected to CMA/DAPI fluorescent banding procedure (Guerra; 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) with $MgCl_2$ 2.5M for 1 hour. Chromosomes were stained with DAPI (1.5 μg/mL) in 9 μL of Vectashield. The slides were stored at 4 ºC for at least three days prior to evaluation.

Fluorescent *in situ* hybridization (FISH)

Sequences of 35S rDNA (pTa 71 *Triticum aestivum* L.) and telomeric DNA, labelled by nick translation PCR (Ijdo et al., 1991) with biotin and digoxigenin, respectively, were used as probes for FISH. A hybridization mixture containing 50% formamide, 2 × SSC, 10% dextran sulfate and approximately 40 ng/µL of the 35S and telomeric probes was applied on chromosome preparations. Slides were placed on a hotplate at 80 °C for 3 min to denature the chromosomes/probes and then kept in a humid chamber overnight at 37 °C (Křivánková et al., 2017). Post-hybridization washes were done 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.

Fluorescent microscopy analysis

The chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI)/Vectashield®. The slides were evaluated in 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.

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 per sample were used. For each plant, three repetitions were performed. The DNA amount of *Solanum lycopersicon* cv. "*Stupické*" was used as a standard reference (2C=1.96 pg). Samples were macerated in a Petri dish containing 1mL of cold buffer WPB (woody plant buffer) (Loureiro et al., 2007) to obtain a nuclear suspension, to which 25µL propidium iodide (1mg/mL) 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 software Cell Quest (Becton, Dickinson and Company, San Jose, CA, USA). The mean nuclear DNA content was estimated with the software WinMDI 2.8 (2009). Based on the nuclear DNA content means, clones were grouped by ploidy level within each genotype and analysis of variance and 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, at 140th day. 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 from the median region of the leaves and the epidermis was dissociated by immersion in 10% sodium hypochlorite for 12-24 hours. Subsequently, the preparation was stained with 5% safranin and semipermanent slides were mounted with glycerin medium. Observations were made in a light microscope (Carl Zeiss, Axio Lab A1) equipped with a camera (Axio Cam), using the software Axio Vision under a total magnification of 400x and real visible field of 0.04843 mm^2.

For the 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 (PD) and equatorial (ED) diameters. Stomatal density (SD - number of stomata per unit area) and stomatal functionality (SF - polar diameter/equatorial stomata diameter ratio) were estimated (Castro; Pereira; Paiva, 2009). The morphometric data was obtained using ImageJ software (Schneider; Rasband; Eliceiri, 2012).

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

An 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

Chromosomal counts

All metaphases from clone F of *E. grandis x E. urophylla* exhibited 2n=44 chromosomes (Figure 1A, Table 1). In the remaining clones, cells with 22, 27, 30, and 40 chromosomes were also observed, which demonstrated the occurrence of mixoploidy (Figure 1B and 1C, Table1). The diploid clone, used as control, presented invariably 22 chromosomes. Many metaphases from diploid and polyploidized clones displayed supposed chromosomal fragments, resulting

from breaks or gaps in some chromosomes (Figure 1D, 1E and 1F). These presumable fragments were differentiated by their size and morphology compared to intact chromosomes. Break or gap sites were identified by CMA+ bands (regions rich in CG) (Figure 1E), co-localized with 35S rDNA sites, as shown by FISH (Figure 1F). The telomeric probe did not show fluorescent labeling in one of the terminal regions, confirming that the fragment is actually the detached Nucleolus Organizer Region (NOR) segment (Figure 1F).

The estimated nuclear DNA content was 1.57 pg for the diploid clone and 2.15-2.74 pg for the polyploids. The polyploids were grouped into two statistically different groups: one containing clones A, B, and F, and the other with clones C and E (Table 1).

It is worth mentioning that the polyploid plants were produced by the company using the same polyploidization induction protocol.

Table 1 Chromosomal counts and DNA content (2C value) in picograms (pg) of *E. grandis x E. urophylla*, diploid and polyploid clones.

CLONES		**Number of metaphases**			**Ploidy level**	**DNA content (pg)**
		22 chromosomes	**44 chromosomes**	**Mixoploids**		
Diploid		30	0	0	2x	1.57 **c**
Polyploid	**A**	1	24	5	4x	2.64 **a**
	B	0	27	3	4x	2.67 **a**
	C	0	28	2	4x	2.15 **b**
	E	0	24	6	4x	2.40 **b**
	F	0	30	0	4x	2.74 **a**

Means followed by the same letter belong to same group according to the *Scott Knott* test (1974), at the probability of 5%.

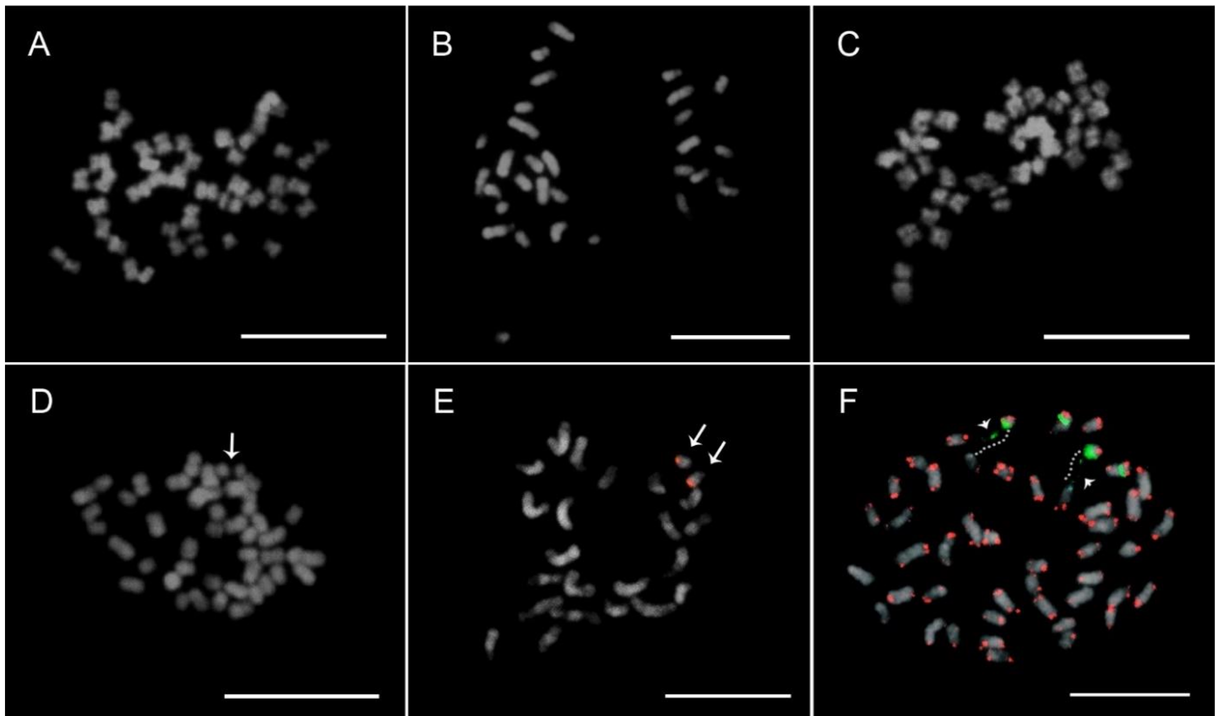

Figure 1 C-metaphases of *E. grandis x E. urophylla* clones. The images A, B, C, D, and F were obtained from polyploid plants and the image E from a diploid plant. A. 44 chromosomes; B. 30 chromosomes; C. 40 chromosomes; D. 44 chromosomes + 1 detached NOR segment (arrow); E. 22 chromosomes + 2 detached NOR segment (arrows) and CMA bands (orange fluorescence) co-located with 35S rDNA sites; and F. 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.

Stomatal analysis and leaf area

Changes in phenotypic expression between ploidy levels, especially in the stomatal diameter of the clones' leaves, were observed. Polyploids presented larger stomatal diameters (Figure 2). In some cases, there was no significant difference between diploids and polyploids (Table 2), as effect of mixoploidy.

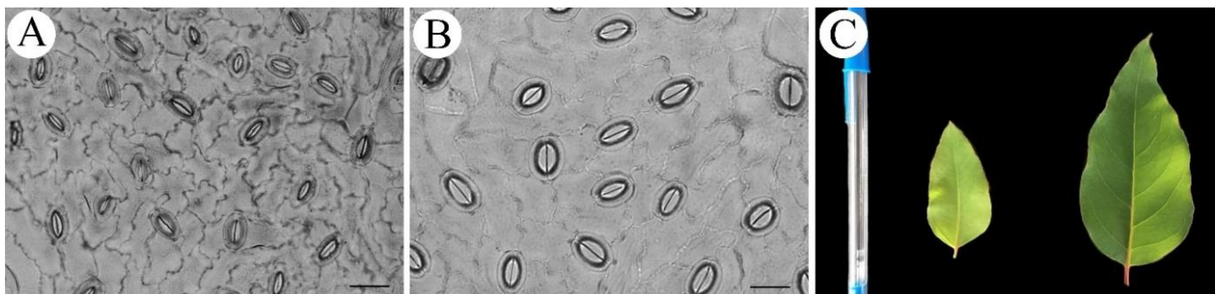

Figure 2 Paradermic sections of the abaxial surface of leaves of *E. grandis x E. urophylla* diploid (A) and tetraploid (B) clones (Bar: 50 µm). Leaves 210 days after emergence from diploid eucalypt clones, on the left, and tetraploid, on the right (C).

The diploid and tetraploid clones B and C presented larger stomatal density, around 488.7 stomata/mm², with no significant difference among them. The largest polar diameter values were observed in clones C, E, and F (18.3 µm) and the largest equatorial diameter values were found in polyploid E (11.7 µm). Stomata presented an ellipsoid shape in all clones, as the equatorial diameter was smaller than the polar diameter (Table 2). Regarding stomatal functionality (polar diameter / equatorial diameter), the diploid clone presented larger values (2.65), which were statistically different from polyploids (Table 2).

Table 2 Stomatal density (SD), polar diameter (PD), equatorial diameter (ED), stomatal functionality (SF) of the abaxial face of *E. grandis x E. uroplhylla* leaves and leaf area of the diploid and polyploid clones (A, B, C, E, and F).

CLONES		SD (st/mm²)	PD (µm)	ED (µm)	SF	LEAF AREA (cm²)
Diploid		493.76 **a**	17.13 **b**	6.49 **c**	2.65 **a**	12,313.07 **b**
Polyploid	**C**	474.14 **a**	17.65 **a**	9.70 **b**	1.82 **b**	29,521.07 **a**
	F	395.68 **b**	18.54 **a**	10.47 **b**	1.77 **b**	28,688.67 **a**
	A	420.97 **b**	16.78 **b**	10.38 **b**	1.62 **c**	28,085.93 **a**
	E	399.55 **b**	18.72 **a**	11.69 **a**	1.60 **c**	24,864.73 **a**
	B	498.14 **a**	15.73 **b**	9.90 **b**	1.60 **c**	22,023.40 **a**

Means followed by the same letter belong to same group according to the *Scott Knott* test (1974), at the probability of 5%.

Leaf area increased significantly as a function of ploidy levels, with averages of 26,636.76 cm² and 12,313.07 cm² for polyploids and diploids, respectively, and no significant differences were found among polyploid clones. These values represent an increase of 46% for polyploidized clones (Table 2, Figure 2).

DISCUSSION

The cytogenetic analyses confirmed metaphases with 44 chromosomes and the occurrence of mixoploids, with chromosome numbers varying from 22 to 44 in plants treated with the same polyploidizing agent. The chromosome number and morphology observed in diploid plants are consistent with previous descriptions reporting invariably 2n=22 chromosomes for *Eucalyptus* species (Haque, 1984; Matsumoto et al., 2000; Bachir; 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 are originated in nature and their relative fertility.

The position of CMA+ bands, co-located with 35S rDNA sites in two chromosomes of the diploid *E. grandis x E. urophylla* (2n=2x=22) clone was also previously observed by Ribeiro et al., (2016). This site constitutes the RON and often becomes detached from the chromosomes

due to extension and late condensation, but it does not promote fragmentation and loss of DNA (Gustafson; Dera; Petrovic, 1988; Flavell, O`Dell; Thompson, 1988; Sakowicz; 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 behavior of extended 35S rDNA sites may mislead chromosome counts, especially in species with small chromosomes and 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 for identifying mixoploid plants.

The mixoploidy observed in artificially polyploidized *Eucalyptus* plants is an expected event since antimitotic agents act on explant cells in different cell cycle stages, resulting in ununiform tissues (Pereira et al., 2012). In a study by Matriz (2008), six out of 17 presumably polyploid *E. urophylla* plants produced with colchicine treatments were identified as tetraploids and 11 were mixoploids. In five *E. grandis* individuals regenerated from seed treated with colchicine, one was tetraploid and four were mixoploids. In a different study, 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 polyploidizing agents 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 *E. dunnii* clones (Castillo et al. 2020).

The variation in chromosome number caused by mixoploidy is not desirable since it may compromise the fertility and uniformity of the plants depending on its extension (Pereira et al., 2012), although the use of mixoploid plants in the field is still feasible. Given that mixoploidy is related to variability, it can be exploited with selection under field conditions. Thus, the genetic and cytogenetic monitoring of polyploidized genotypes is necessary for a better understanding of polyploidization and the commercial use of the plants subjected to this process.

The flow cytometry results indicate the occurrence of polyploidy and evidenced differences in DNA content among polyploids, which is consistent with the mixoploidy identified in the chromosome counts. The DNA content estimates in diploid plants of *E. grandis x E. urophylla* found in our study (1.57 pg) differ in 0.4-0.5 pg from descriptions for other diploids, such as *E. globulus* (1.13 pg) (Marie; 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 some technical factors, such as calibration of the cytometer, and differences in reference standard, plant material, extraction buffer, and fluorochrome used (Timbó et al., 2012). Moreover, the divergences in the 2C value among the mentioned studies and the variation observed among polyploid clones in our study, may be due to differences in the DNA content among cells. The genome organization in plants is dynamic and, more so in recent polyploids, which often undergo events that may alter the DNA content, such as loss and gain of repetitive sequences and activation of transposable elements (Jones; Hegarty, 2009).

Regarding the anatomical traits, the 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 compared to diploids. Both studies confirm that stomatal analysis can be used as an indirect method for identifying ploidy levels in *Eucalyptus*, which has also been shown for other plants (Magallanes et al., 1996; Omidbaigi et al., 2012; Feng et al., 2017; Chaves et al., 2018).

The increase in stomata size was accompanied by a lower stomatal density in most polyploid clones. Diploid plants displayed a larger stomatal density, although statistically similar to the polyploid clones B and C. The smaller number of stomata per mm² in tetraploids was also observed in *E. dunnii* compared to diploid plants (Souza, 2016). Similarly, Moura et al. (2020) found that the average stomata size in tetraploid clones of *E. urophylla* was larger than in diploids.

The increase in DNA content may interfere in the dimension of vegetative organs and alter tissue structure (Hodgson et al., 2010; Balao et al., 2011; Del Pozzo et al., 2015). However, our findings suggest that the more elliptical shape of the stomata in diploid plants was advantageous for stomata functionality. In this sense, it is assumed that diploid plants can be more efficient in opening and closing the stomata, resulting in a reduction in water loss through transpiration. According to Khan et al. (2003), stomata shape is an adaptive trait for plants, as the ellipsoid shape has a smaller equatorial diameter, which results in increased functionality. Polyploids on the other hand tend to lose more water due to their larger stomata. In this case, stomata opening and closing mechanisms during transpiration are slower. Changes in stomatal conductance due to larger stomata and lower density can also increase the 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 diploids. Likewise, Souza (2016) and

Fernando et al. (2019) found an increase in leaf structure and area in tetraploid plants of *E. dunnii* and *E. polybractea*, respectively, compared to diploids.

All these anatomical and morphophysiological events result from the increase in gene copies and transcription products, which are the main evidence of the efficacy of polyploidization (Satler et al., 2016). Such traits allow the differentiation of diploids and polyploids, although it is necessary to confirm the agronomic performance of polyploid individuals in the field to validate the importance of this biotechnological technique in *Eucalyptus* breeding.

CONCLUSIONS

The chromosome counts and estimation of DNA content allow the identification of artificially tetraploidized and mixoploid clones of *E. grandis x E. urophylla*.

Stomata size, stomatal functionality and leaf area may be used as indirect parameters to detect ploidy levels, allowing the discrimination of diploid and polyploid plants.

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ARTICLE 2 – POLYPLOIDY AS A STRATEGY TO IMPROVE THE INDUSTRIAL QUALITY OF EUCALYPT WOOD

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ORIGINAL

Polyploidy as a strategy to improve the industrial quality of eucalypt wood

Thaise da Silva Souza[1] · **Marcela Faralhi Daolio**[1] · **Fábio Akira Mori**[2] · **Magno Antonio Patto Ramalho**[1] · **Fabiana Bombonato Mingossi**[3] · **Alexandre Alves Missiaggia**[3] · **Vânia Helena Techio**[1]

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Abstract

There are many studies describing the physical and anatomical properties of eucalypt wood, but studies comparing the performance of diploid and polyploid clones in terms of wood anatomy and wood quality are scarce. In this context, this study aimed to compare the performances of *Eucalyptus* diploid and polyploid clones with the same genetic origin by analyzing the physical and anatomical properties of the wood to extend the information on wood quality for pulp and paper production. Woods samples from eucalypt hybrids (*E. grandis* x *E. urophylla*) from experimental plantations of Suzano S.A. were used. Moisture content, basic density and fiber size were determined. The data related to these variables were tested by analysis of variance. Moisture content in polyploids was 4% higher than in diploids. The mean basic density in diploid clones was 13% higher than in polyploid clones, although there was no significant difference between one of the evaluated clones. The mean fiber length in polyploid clones was 18% higher than in diploid clones. No significant differences were found in the wood quality indexes for cellulosic pulp production. Synthetic polyploid clones of eucalypt presented lower basic density and fibers with higher length and wall thickness when compared to diploids, indicating the potential use of polyploids in eucalypt pulp and paper production.

✉ Vânia Helena Techio
vhtechio@ufla.br

[1] Department of Biology, Federal University of Lavras, Lavras, Minas Gerais State, Brazil

[2] Department of Forest Sciences, Federal University of Lavras, Lavras, Minas Gerais State, Brazil

[3] Suzano S.A., Technology Center, Jacareí, São Paulo State, Brazil

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Introduction

In the eucalypt forest-based industry, the pulp production for paper is noteworthy (Carvalho et al. 2015). Brazil is a reference in pulp production, with 19.2 million tons produced last year, of which 86% comes from short fiber pulp originated from eucalypt planted forests (DEPEC-BRADESCO 2018). These numbers place the country as the fourth largest cellulose producer of all types and the world's largest producer of cellulose from *Eucalyptus* species (IBÁ 2018).

Although eucalypt breeding in Brazil is a huge success, the growing demand for wood, especially for cellulose production, requires even higher productivity. Since the clone productivity levels are already high, further progress through selective breeding can be time-consuming. An interesting alternative is polyploidy induction (Han et al. 2011), mainly because there is evidence of numerous plant species that undergo polyploidy or endopolyploidy events in their evolutionary process (Myburg et al. 2014). Polyploids have double the amount of DNA (Pereira et al.2012) and are usually larger and more robust than their diploid relatives (Sattler et al. 2016). This increase in genome size has led, in particular, to the wider adaptation of plant species.

The induction of synthetic polyploids has already been tested for *Eucalyptus* (Lin et al. 2010; Han et al. 2011). More recently, promising results have intensified the research on eucalypt polyploidy in Brazil.

Much of the work on polyploids in forest species has focused on growth-related variables (Bona et al. 2016; Biondo et al. 2005). However, information comparing wood anatomy between diploid and polyploid plants is still poorly explored.

In this context, the present study aimed to compare the performance of *Eucalyptus* diploid and polyploid clones with the same genetic origin by analyzing the physical and anatomical properties of the wood to provide more information on wood quality for pulp and paper production.

Materials and methods

All test samples used in this study were 36-month-old eucalypt hybrids (*E. grandis* x *E. urophylla*) from experimental plantations of Suzano S.A. at Caçapava-State of São Paulo. Diploid and polyploid clones were used. The polyploid clones (clones A, B and C) were produced via artificial tetraploidization by the company. The experiment consisted of 13 treatments and 39 sampled individuals (three individuals per treatment—three diploids, five clone A polyploids and five clone B polyploids). Each polyploid clone presented five different polyploidy events, and each event was represented by three plants. Diploid clones A and B were used as control group for polyploid clones A and B. The experiments were implemented in 2016, in a randomized block design, with 100 treatments (diploids and polyploids), plots of ten plants and 20 repetitions. The plant spacing used was 3×2.5 m.

The samples consisted of stem discs removed at 1.30 m from the ground. Each disc was cut into four wedges.

Moisture content and basic density

The determination of moisture content, basic wood density and fiber dimensions was performed at the Wood Anatomy Laboratory at the Department of Forest Sciences of the Federal University of Lavras. For the moisture content and basic density assays, a DBH (diameter at breast height) disc was used according to ABNT NBR 11941-02 (Associação Brasileira de Normas Técnicas-ABNT 2003). The samples were saturated in water to determine the basic density, and the green volumes were measured by immersion and then drying in a drying chamber at 105 °C for 48 h until the mass was fully stabilized and dry.

The moisture content (MC) and basic density (BD) were determined according to ABNT NBR 7190/1997 (ABNT 1997). These variables were not measured immediately after felling, so an initial mass of 39 specimens was specified before the measurement procedures.

Fiber dimensions

The maceration method was performed according to Franklin (1945) to evaluate fiber traits, using a 1:1 hydrogen peroxide and acetic acid (v/v) solution, in a greenhouse at 60 °C for 24 h or until the solution was clear and the wood pieces turned whitish. After cooling, the material was carefully washed under running water. Semi-permanent slides were prepared with 1% (w/v) safranin in 50% ethanol (Berlyn and Miksche 1976). The slides were mounted on glycerin diluted in distilled water.

Anatomical measurements were taken using photomicrographs obtained using a 3-megapixel Pixelink digital camera, coupled with an Olympus microscope. Measurements of fiber length (L), width (W), lumen diameter (d) and, indirectly, cell wall thickness (t) were taken using the "WinCell" image analyzer software; the trait "W" corresponds to the total fiber diameter. Forty measurements of each individual were used.

Relations between fiber dimensions

The following indices of wood quality for pulp production were calculated according to Foelkel and Barrichelo (1975): Runkel index ($RI = 2t/d$), Flexibility Coefficient ($FC = (d/L) \times 100$), Envelope Index ($EI = L/W$) and Wall Fraction $WF = (2t / W) \times 100$).

Statistical analysis

The normality of data and homogeneity of variance were confirmed by the Shapiro–Wilk test ($P < 0.05$) and Bartlett test ($p < 0.05$), respectively.

A fully casualized design (FCD) was used, and the analysis of variance was performed using the following statistic model (Eq. (1)):

$$Y_{ij} = \mu + C_i + e_{j(i)} \tag{1}$$

where:

Y_{ij}: observed value for clone i associated with plant j;

μ: mathematical constant;

C_i: effect of clone i (A, B, C);

$e_{j(i)}$: mean error.

The source of variation clone was partitioned into 'between clones' (diploid clone (*D*) and polyploid clone (*P*)) and 'between events' within polyploid clone (*E*/*P*). The estimate accuracy (*rgg'*) was obtained for the treatments that presented performance variation, using the following estimator:

$$rgg' = \sqrt{1 - \left(\frac{1}{F}\right)}$$

where F is the mean square of the source of variation divided by the mean squared error.

The mean performance of the clone diploids and polyploids was ranked by the Scott & Knott (1974) test.

All statistical analyses were performed using the software R (R Core Team 2016).

Results

Moisture content and basic density

Analysis of variance displayed performance variation between diploid and polyploid clones (Fig. 1) in terms of wood basic density and moisture content. Basic density also varied within polyploid clones for density (Table 1). Moisture content in polyploids was 4% higher than in diploids (Table 1). The mean basic density in diploid clones (0.490 g/cm^3) was 13% higher than in polyploid clones (0.426 g/cm^3) (Table 1). The accuracy (*rgg'*) for these traits was of high magnitude (0.97).

Fiber dimensions

Significant differences ($P \leq 0.05$) in fiber length, outer diameter, inner diameter and cell wall thickness were observed between diploid and polyploid clones (Table 2). The mean fiber length ranged from 578.24 to 799.36 µm; fiber width ranged from 16.56 to 23.72 µm; diameter values ranged from 10.28 to 15.42 µm; and cell wall thickness ranged from 3.10 to 4.54 µm (Fig. 2). The mean fiber length in polyploid clones (744.70 µm) was 18% higher than in diploid clones (613.53 µm); the outer diameter, inner diameter and wall thickness of the fibers were also larger in polyploid clones than in diploids (Table 2). The estimate accuracy (*rgg'*) was of high

Fig. 1 *E. grandis x E. urophylla* seedlings at 60 days; tetraploid on the left and diploid on the right

Table 1 Summary of analysis of variance and means for wood basic density (BD) and moisture content (MC) of *E. grandis x E. urophylla* in 13 treatments, diploids and polyploids

SV	df	MS	
		BD (g/cm^3)	**MC (%)**
D vs. *P*	1	28.073**	0.91**
P	1	20.957**	0.01
E/*P*	8	2.005	0.07
Error	28	1.364	0.06
Mean	Polyploid A	0.453 b	8.96 a
	Polyploid B	0.400 c	8.92 a
	Diploid A	0.475 b	8.72 b
	Diploid B	0.447 b	8.43 b
	Diploid C	0.550 a	8.59 b
	Polyploids	0.427 b	8.94 a
	Diploids	0.490 a	8.58 b

Means followed by the same letter belong to the same group by the Scott Knott test (1975) at 5% of confidence. The source of variation (SV) clone was partitioned into 'between clones' [diploid clone (*D*) and polyploid clone (*P*)] and 'between events' within polyploid clone (*E*/*P*)

*significant at 5% of confidence, by F-test

**significant at 1% of confidence, by F-test

Table 2 Summary of analysis of variance and means for wood fiber length (*L*), width (*W*), outer diameter (*D*) and inner diameter (*d*) of *E. grandis x E. urophylla* in 13 treatments, diploids and polyploids

SV	df	MS			
		L (μm)	*W (μm)*	*D (μm)*	*d (μm)*
D vs. *P*	1	119,126,00**	5.33**	107.70*	33.00*
P	1	1000	0.00	7.90	7.10
E/*P*	8	3251.87*	0.33	1.55	2.94
Error	28	1057.93	0.19	3.77	4.17
Mean	Polyploid A	750.48 a	4.09 a	22.11 a	13.92 a
	Polyploid B	738.93 a	4.07 a	21.09 a	12.94 a
	Diploid A	627.80 b	3.38 b	18.55 b	11.80 b
	Diploid B	578.24 b	3.14 b	16.56 b	10.28 b
	Diploid C	634.54 b	3.10 b	17.85 b	11.66 b
	Polyploids	744.70 a	4.08 a	21.60 a	13.43 a
	Diploids	613.53 b	3.20 b	17.65 b	11.25 b

Means followed by the same letter belong to the same group by the Scott Knott test (1975) at 5% of confidence. The source of variation (SV) clone was partitioned into 'between clones' [diploid clone (*D*) and polyploid clone (*P*)] and 'between events' within polyploid clone (*E*/*P*)

*significant at 5% of confidence, by F-test

**significant at 1% of confidence, by F-test

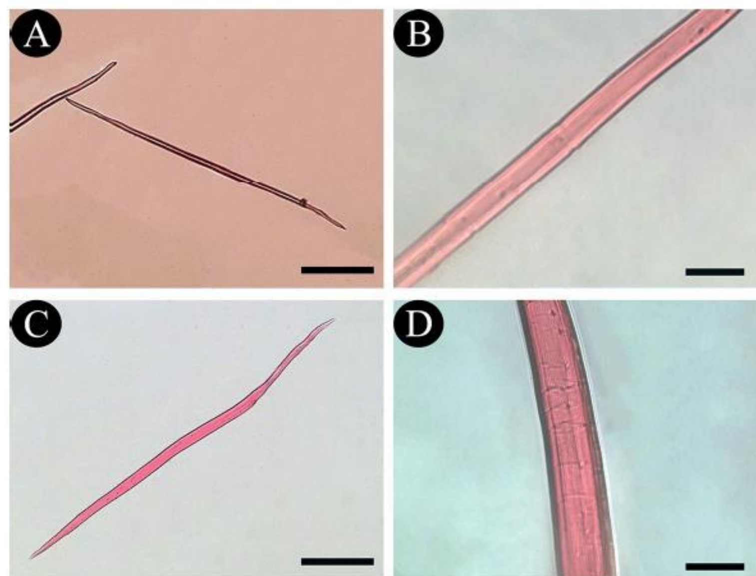

Fig. 2 Fibers of *E. grandis x E. urophylla* diploid (A, B) and polyploid clones (C, D). Bars—A, C: 100 μm; B, D: 20 μm

magnitude for all traits (above 0.93). There were also differences in fiber length between polyploid clones of distinct polyploidy events.

Relations between fiber dimensions

No significant differences ($P \geq 0.05$) were found in the wood quality indexes for cellulosic pulp production (Runkel index, flexibility coefficient, envelope index and wall fraction), which are based on fiber dimension parameters (Table 3).

Runkel index means ranged from 0.52 to 0.76. The flexibility coefficient means ranged from 57.70 to 66.05%, and values between 31.29 and 36.46 were found for the envelope index. Wall fraction means ranged from 33.95 to 42.30%.

Discussion

Although there are many studies describing the physical and anatomical properties of eucalyptus wood (Baldin et al. 2017; Boschetti et al. 2015; Kumar et al., 2011; Gomide et al. 2005), studies comparing the performance of diploid and polyploid clones in terms of wood anatomy and wood quality are scarce. Given the time required to induce polyploidy and the small sample size used in our analyses, additional studies will be carried out to complement the analyses and confirm the results.

The high accuracy estimates ($rgg' > 0.93$) found in this study indicate good repeatability of the information among the different repetitions, which means that the statistical analyses were highly reliable. The analysis of variance showed performance variation between diploid and polyploid *E. grandis x E. urophylla* clones in terms of basic density, moisture content, fiber length, width and outer and inner diameter.

Table 3 Summary of analysis of variance and means for wood quality indices of *E. grandis x E. urophylla* in 13 treatments (RI: Runkel index, FC: flexibility coefficient, EI: envelope index and WF: wall fraction), diploids and polyploids

SV	df	MS			
		RI	**FC (%)**	**EI**	**WF (%)**
D vs. *P*	1	0.005	12.00	1.00	11.00
P	1	0.011	17.00	7.00	18.00
E/*P*	10	0.024	31.12	8.75	31.50
Error	26	0.018	24.36	5.75	23.86
Mean	Polyploid A	0.61	62.67	34.19	37.28
	Polyploid B	0.65	61.16	35.18	38.84
	Diploid A	0.58	63.32	34.20	36.68
	Diploid B	0.68	61.07	35.15	38.93
	Diploid C	0.53	65.23	35.72	34.77
	Polyploids	0.63	61.92	34.69	38.06
	Diploids	0.60	63.21	35.02	36.79

The source of variation (SV) clone was partitioned into 'between clones' [diploid clone (*D*) and polyploid clone (*P*)] and 'between events' within polyploid clone (*E*/*P*)

There is a strong correlation between the volumetric performance of eucalypt clones at 3 and 5 years (Rezende et al. 2014). However, wood anatomy traits may change with age, mostly since most commercialized raw material is made of juvenile wood. Juvenile wood is anatomically characterized by a progressive increase in cell size and corresponding changes in its shape, structure and arrangement in successive growth rings (Ballarin and Palma 2003). Thus, the values and inferences resulting from this study might change once the tree cutting cycle is completed, given that the evaluated samples were only 36 months old. Nevertheless, relative continuity of the different responses and maintenance of ploidy levels are expected.

Basic density usually increases with age (Meneses et al. 2015), and a few studies are reporting this trend (Kumar et al. 2011; Neves et al. 2013). This condition is associated with increased cell wall thickness and reduced vessel element frequency, as latewood is progressively formed (Sette Junior et al. 2012). The same logic is valid for fiber morphology since wood properties stabilize over time.

Moisture content and basic density

High wood moisture gradients are among the causes of drying defects, notably those characterized by warping and cracking (Oliveira et al. 2005). In this work, the values found for this trait were low compared to other studies, ranging from 8.43 to 9.17%, which is an advantage in wood drying processes. The analyses showed a significant difference between the samples evaluated for wood moisture content, in which polyploids were relatively higher than diploids.

Wood basic density is recognized as one of the most important parameters to assess wood quality since it is easy to determine and related to other traits (Shimoyama and Barrichello 1991). The values found for basic density in this work are consistent with the literature, ranging from 0.368 to 0.550 g/cm^3, with a higher mean for diploid treatments (0.490 g/m^3) than polyploids (around 0.427 g/m^3). However, when the performance of polyploids is compared to the diploid control, the same is not observed. The mean performance found for polyploid A is not significantly different from that of the diploid control (diploid A), whereas the values found for polyploid B are lower than its diploid control (diploid B). However, the basic density of polyploids is lower than that of diploid C clones (Table 1). These values are considered intermediate and suitable for pulp production. Oliveira et al. (2005) found basic density values of 0.540 and 0.490 g/cm^3 for *E. urophylla* and *E. grandis*, respectively. Another study reported values of 0.559 g/cm^3 for *E. urophylla* and 0.479 g/cm^3 for *E. grandis* (Ribeiro and Zani Filho 1993).

The lower density in polyploids indicates a better accommodation of fibers in paper formation, despite reducing the packing in the digestor. This variation may or may not be advantageous for the industry. Higher density values reduce the specific wood consumption and the variable cost of pulp production, as the specific consumption represents the amount of solid wood, in volume (m^3), required to produce one ton of pulp. However, lower density pulps are more suitable for manufacturing paper for printing and writing (Santos and Sansígolo 2007).

Fiber dimensions

Fiber length in eucalypt varies from 750 to 1400 µm and is the cellular element most related to wood density and mechanical strength (Silva et al. 2007). In a study with two eucalypt clones, Melo (2004) found fiber length means ranging from 1000 and 1090 µm. Lower values were found in this study due to the young age of the analyzed samples (36 months). The evaluated samples may have short fibers, a trait that results in a paper with better ink absorption and smoother sheets, making the wood desirable for the production of paper for printing (Rodrigues 2016). Shorter fibers provide better paper sheets, with lower physical and mechanical properties than paper produced from long fiber pulp (Bassa et al. 2007; Alves et al. 2011).

In general, the fiber length is longer in polyploids than in diploids. This anatomical variable is directly proportional to wood resistance. Thus, the longer the fiber length, the greater the resistance, which allows the inference that wood from synthetic polyploids has more resistant fibers than diploid plants. Paper quality is highly influenced by morphological aspects of the fiber and their fraction in the pulp (Gomide et al. 2005). Determining the quantitative parameters of fibers is crucial for predicting pulp behavior under operational conditions. Fibers are the most abundant anatomical element in wood and features like length, wall thickness and width directly affect the yield and quality of the final product (Baldin et al. 2017).

According to the classification proposed by Tomazello Filho (1985), the wood fibers of the evaluated samples are considered narrow. This same study reported similar values for *E. grandis* (23 µm) and *E. pilularis* (23 µm). Similar mean values for fiber lumen diameter were also found in *E. gummifera* (9.5 µm) and *E. pilularis* (11.6 µm).

The fiber wall thickness measures found in this study are consistent with mean values observed in hardwoods, ranging from 3 to 5 µm (Miranda and Castelo 2012). These numbers are usually superior in polyploid when compared to diploids. Fibers with thicker walls require higher energy consumption in the pulping process and confer less resistance to paper sheets (Rodrigues 2016). Thicker walls tend to increase the stiffness and roughness of the fiber, which generates thicker sheets, suitable for use in absorbent papers of high specific volume, also known as tissue paper (Mokfienski et al. 2008). Nonetheless, a gelatinous layer tends to detach from the cell wall, which may hinder fiber refinement and compromise paper quality (Pilate et al. 2004).

Relations between fiber dimensions

The estimated indices indicate the quality of *E. grandis x E. urophylla* wood fibers for pulp and paper production. Relations between fiber dimensions are widely used in papermaking and are more important than isolated measures since they impact the final product properties (Menegazzo 2012).

The Runkel index (RI) represents the fiber bonding capacity, and it includes five classes: excellent paper fiber (≤ 0.25), very good (0.25–0.50), good

(0.50–1.00), regular (1.00–2.00) and inappropriate (> 2.00) (Foelkel and Barrichelo 1975). The measures found in this study for both diploid and polyploid clones (0.52–0.76) fall into the third class, i.e., they are considered of good quality for paper production. Similar RI values for *E. grandis x E. urophylla* wood were reported by Santos (2005) (0.47–0.60) and Menegazzo (2012) (0.60 and 0.48).

The flexibility coefficient (FC) is related to paper tear strength and burst strength (Silva et al. 2012). Values greater than 50 indicate good quality paper (Foelkel and Barrichelo 1975). The coefficients found in this study (57.70–66.05%) fit this parameter and indicate wood fibers of medium flexibility, with a good fiber–fiber union. These results were consistent with the FC values of *E. grandis x E. urophylla* wood reported by Santos (2005) (62.79–67.82%) and Menegazzo (2012) (62.8 and 67.62%).

The envelope index (EI) is the ratio between fiber length and width and is related to tear strength, increasing EI. The mean for *Eucalyptus badjensis*, reported by Vieira et al. (2017), is 65.72. In a previous study, Menegazzo (2012) found mean values of 49.32 and 45.44 *for E. grandis* and *E. urophylla*, respectively. The variation found in this study was lower (31.29–36.46) and indicates wood samples with medium resistance.

The wall fraction is a measure of fiber stiffness associated with burst strength and flexibility for fiber bonding (Boschetti et al. 2015). In papermaking, values below 40% are desired (Silva et al. 2012) and this is observed for most treatments assessed in the present study (Table 3). Wall fraction measures higher than 40% indicate fibers are too rigid, with low flexibility and difficulties in their interconnection (Rodrigues 2016). This parameter has a strong correlation with tear strength and weak correlation with tensile strength, burst strength and apparent specific gravity (Foelkel and Barrichelo 1975). Considering these aspects, the samples evaluated in this study have median stiffness fibers.

Conclusion

Synthetic polyploid clones of *E. grandis x E. urophylla* presented lower basic density and fibers with higher length and wall thickness than diploids, which improves fiber accommodation in the paper formation and increases its strength, indicating the potential use of polyploids in eucalypt pulp and paper production.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest.

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ARTICLE 3 – POTENTIAL USE OF POLYPLOID EUCALYPT IN FORESTRY

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POTENTIAL USE OF POLYPLOID EUCALYPT IN FORESTRY

Thaise da Silva Souza, Fabiana Rezende Muniz, Vânia Helena Techio, Alexandre Alves Missiaggia, Magno Antonio Patto Ramalho

ABSTRACT

Polyploidy induction has been used as a strategy to select new clones, potentially increasing the genetic progress obtained by *Eucalyptus* breeding in Brazil. Genetic variation occurs among polyploids produced by the chromosome duplication of a diploid clone, which can affect their performance. The present study aimed to evaluate the performance of polyploid *Eucalyptus* clones under field conditions to verify if they are more adapted and stable for forest exploration and if polyploid performance depends on its diploid. The evaluations were performed on 80 synthetic tetraploid clones obtained, in equal number, from two diploid clones of *E. grandis* x *E. urophylla* hybrids. The experiments were conducted in four environments in São Paulo and Mato Grosso do Sul States, Brazil. Measurements of DBH (diameter at breast height) and MAI (mean annual increment) of three-year-old trees were taken. Results showed that the clones x environments interaction was relatively expressive, although predominantly simple in many cases, indicating that the ranking of clones was similar among the evaluated sites. More adapted and stable clones were identified, although in most cases their performances were lower than diploids. The performance of polyploid clones was not affected by the performance of their diploid. The results found were promising and indicate that the selection of polyploids that outperform diploid clones should be pursued, although there are still many questions regarding the use of polyploidy in *Eucalyptus.*

Keywords: Eucalypt breeding, genotype by environment interaction, adaptability, stability, polyploidization.

INTRODUCTION

Despite the enormous success of *Eucalyptus* yield in Brazil (Ramalho et al., 2021), the growing demand for wood, especially for pulp production, requires higher productivity levels. One of the alternatives to meet current demands is the production of new clones with higher performances. However, since wood/pulp productivity is already high, it is not easy to achieve greater gains from selection. Therefore, other strategies are being discussed. One example is the reduction of the time needed to obtain new clones employing a speed breeding technology (Watson et al., 2018), although a significant acceleration has already been obtained with the induction of early flowering (Alfenas et al., 2004).

An alternative approach being used, although with no proven efficacy yet, is to evaluate the clones without the initial progeny test, using what is called clonal progeny test (Resende, 2002). In this case the seeds obtained in the recombination lot are cloned in the nursery soon after germination (Ramalho et al., 2021).

Another option is the use of molecular biology tools, especially molecular markers. This technology has been employed for some time in eucalypt, and its success is still being assessed. Nonetheless, new perspectives are being created with genomic selection as genome sequencing becomes more accessible (Ballesta et al., 2020; Moura et al., 2019; Muller et al., 2017; Grattapaglia, Resende, 2011).

Lastly, the artificial induction of polyploidization in eucalypt is another viable option to increase gains (Castillo et al., 2020; Fernando et al., 2019; Han et al., 2011), as there are reports of alterations in wood quality in *E. grandis x E. urophylla* tetraploids (Souza et al., 2021). Polyploid induction in plants is widely used for producing novel phenotypes, some of which can be of value for breeding programs (Fernando et al., 2019).

The occurrence of variation among clones obtained from the polyploidization of a diploid clone is expected. However, such variation has not yet been evaluated under field conditions and the potential of polyploid clones regarding their adaptation and stability under growing conditions have not been verified.

In this context, the present study aimed to evaluate the performance of polyploid *Eucalyptus* clones under field conditions to verify if they are more adapted and stable than their diploid for forest exploration and if polyploid performance depends on its diploid of origin.

MATERIAL AND METHODS

Data obtained from the evaluation of *E. grandis x E. uroplhylla* diploid and tetraploid clones were provided by the Suzano SA, an important Brazilian eucalypt pulp and paper producer. The polyploid clones were obtained from two operational diploid clones, referred to herein as A and B. From each of these diploid clones, 40 polyploid events were obtained, using the polyploidization protocol established by the company. The clones were evaluated in four environments/sites, comprising two Brazilian States: Mato Grosso do Sul (MS) and São Paulo (SP) (Table 1).

Table 1 Information of the locations where the experiments were carried out.

Experiment/ Site	State	Altitude (m)	Latitude (S)	Longitude (W)
JAC	Jacareí-SP	567	23° 17' 52"	45° 57' 57"
SUL	Capão Bonito-SP	730	23° 59' 53"	48° 21' 01"
TLA	Três Lagoas-MS	318	20° 47' 14"	51° 42' 13"
TLS	Três Lagoas-MS	318	20° 45' 35"	51° 41' 42"

The experiments were implemented in 2015 in a 10 x 10 lattice design, with 20 replicates and plots of a single plant (single tree plot). The treatments consisted of 40 polyploid clones derived from each diploid (A or B), that is, 80 polyploids, the two diploids (A or B), and 18 operational diploid clones from the company used as controls. The spacing used was 3 m x 2.5 m in JAC experiment and 3 m x 2 m in the other experiments. The management applied in each experiment was carried out according to the silvicultural practices adopted in each operational region of the company.

Measurements of the circumference at breast height (CBH), in centimeters, and tree height (h), in meters, were taken from three-year-old plants. Using the CBH and height measures, the diameter at breast height (DBH) was calculated, in centimeters, according to the following estimator:

$$DBH = \frac{CBH}{\pi} \quad (1)$$

Where π corresponds to 3.1416.

The mean annual increment (MAI) of the trees was also measured, in cubic meters per hectare per year:

$$MAI = \frac{V}{3} * k \quad (2)$$

Where *3* represents the age in years and the constant *k* the number of trees per hectare. The age of three years is widely used for clonal selection in eucalypt breeding programs in

Brazil because it correlates well with rotation age (six to seven years) in terms of ranking (Rezende et al., 2014).

The volume (V), in cubic meters per tree, was estimated using the DBH and tree height (h) measures:

$$V = \frac{\pi DBH^2}{40000} * h * f \quad (3)$$

Where f is a factor of generic form used by the company, corresponding to 0.45.

As there was an imbalance in the data obtained from the experiments, mixed model analyses were performed to obtain BLUP means (Best Linear Unbiased Prediction) for each site evaluated (Bernardo, 2020). The effects of clones and blocks within repetitions were considered random, and repetition was considered fixed.

Subsequently, a joint analysis was carried out with the BLUP means for all sites, using the following model:

$$Y_{iq} = \mu + C_i + L_q + CL_{iq} + e_{iq} \quad (4)$$

Where: Y_{iq}: observed value of clone i at site q; μ: constant; C_i: effect of clone i (i =1...100); L_q: effect of site q (q =1...4); CL_{iq}: effect of the interaction between clone i and site q; e_{iq}: mean variance weighted by the degrees of freedom of the errors.

The estimates of DBH and MAI of the different treatments were also subjected to an analysis of variance (ANOVA), following the procedure presented by Steel et al. (1997). The least squares method was employed so that the sums of squares of the treatments could be decomposed as a function of the origin of the clone. The analysis followed the statistical model:

$$Y_{ijk} = \mu + C_i + R_j + B_{k(j)} + e_{ijk} \quad (5)$$

Where Y_{ijk}: observed value of the clone i in block k within the repetition j; μ: constant; C_i: effect of the clone i (i =1...100); R_j: effect of repetition j (j =1...20); $B_{k(j)}$: effect of block k within the repetition j (k = 1...10); e_{ij}: experimental error associated with the plot total Y_{ijk}, with a mean of zero and common variance to all treatments. The source of variation clone (C_i) was decomposed between polyploids of origin A *vs* diploid A. The similar procedure was done for the clones of origin B and the same source of variation was decomposed between controls.

The accuracy (rgg) was estimated from the ANOVA results in each site, using the estimator:

$$rgg = \sqrt{1 - \left(\frac{1}{F}\right)} \quad (6)$$

Heritability (h_q^2) was also estimated for polyploid clones of each origin (q corresponds to A or B), through the following estimator:

$$h_q^2 = \frac{MSC_q - MSE}{MSC_q} \quad (7)$$

Where MSC_q corresponds to the mean square of polyploid clones (A or B) and MSE corresponds to the mean squared error, obtained in the ANOVA involving all treatments.

The lower (LL) and upper limits (UL) of h_q^2 were obtained according to the expressions of Knapp et al. (1985):

$$LL = \left\{1 - \left[\left(\frac{MSC_q}{MSE}\right) . F_{1-\alpha/2\,(DFE;DFC_q)}\right]^{-1}\right\} \quad (8)$$

$$UL = \left\{1 - \left[\left(\frac{MSC_q}{MSE}\right) . F_{\alpha/2\,(DFE;DFC_q)}\right]^{-1}\right\} \quad (9)$$

Where F is the probability distribution of Snedecor with significance α and degrees of freedom associated with each of sources of variation of each polyploid clone A or B (DFC_q) and error (DFE).

Correlation estimates between the pairs of evaluated sites (r_{XY}) were obtained using the following estimator (Ramalho et al., 2012):

$$r_{XY} = \frac{COV_{XY}}{\sqrt{\sigma_X^2\,\sigma_Y^2}} \quad (10)$$

Where COV_{XY} refers to a genotypic covariance between BLUP means involving all the clones evaluated at the sites X and Y, considering that there is no covariance between evaluation sites and clones; σ_X^2 and σ_Y^2 represent the phenotypic variance between the BLUP mean of clones at the sites X and Y.

The clones x environments (G x E) interaction was decomposed into simple and complex parts using the correlation estimates, according to the methodology presented by Ramalho et al. (2012):

$$MS_{CL} = \frac{1}{2}(\sqrt{MS_{CX}} - \sqrt{MS_{CY}})^2 + \sqrt{MS_{CX}\,MS_{CY}}\ .\ (1 - r_{XY}) \quad (11)$$

Where MS_{CL} is the mean square of the clones x environments interaction, MS_{CX} and MS_{CY} are the mean squares of sites X and Y respectively, and r_{XY} is the correlation between the mean performance of clones under the two conditions. The first part of the interaction is referred as the simple part, due to the differences in the genetic manifestation in the two environments/sites, and the second, the complex part, because it changes the classification of

clones in the different experiments and occurs depending on the magnitude of the correlation r_{XY} of the sites, two by two.

The performance of clones at different sites was also assessed according to the methodology of Nunes et al. (2005). For this, means were standardized by site, involving diploid and polyploid clones of populations A and B, using the estimator:

$$z_{iq} = (\bar{y}_{iq} + \bar{y}_{.q})/s_{.q} \quad (12)$$

Where z_{iq} is the standardized BLUP mean of clone i (origin A or B) at the site q, $\bar{y}_{iq}$ is the BLUP mean of clone i (origin A or B) at the site q, $\bar{y}_{.q}$ is the mean of all clones of origin A or B at site q; and $s_{.q}$ is the genotypic standard deviation between the BLUP means of clones of origin A or B at site q. Since z_{iq} admits negative values, a constant was added to the estimates to turn the values positive. Based on this same methodology, estimates of the sums of z_{iq} – reflecting the adaptability of the clones, and the coefficient of variation (CV) – related to the stability of the clones, were obtained.

All the statistical analyses were performed in the R environment (R Core Team, 2020).

RESULTS

The eucalypt clones were evaluated with high accuracy (rgg) in all sites for both traits (Table 2), which was confirmed by the significant difference (P≤0.01) observed among clones in all experiments (Figure 1, Tables 1 and 2, Appendix). Significant differences (P≤0.01) were also observed among controls in all sites. It should be noted that for the DBH trait, the decomposition of the source of variation involving diploids and polyploids A (DA and PA) was also significant (P≤0.05) and the contrast DA *vs* PA was not significant only in the JAC experiment (P>0.05). Among polyploids A, significant differences (P≤0.05) were detected in all sites, except for the TLS experiment. Considering the clones derived from population B significant differences were detected in all cases, apart from polyploids B in TLA experiment. The contrast PA *vs* PB was significant in all sites.

Figure 1 Diploid and tetraploid eucalypt clones at 135 days showing greater initial growth of the polyploid (diploid on the left and tetraploid on the right).

Table 2 Accuracy estimates (*rgg*) of the four experiments/sites for DBH (cm) and MAI (m^3 ha^{-1} $year^{-1}$) of three-year-old eucalypt plants.

Site*		JAC	SUL	TLA	TLS
rgg	**DBH**	0.96	0.97	0.99	0.99
	MAI	0.96	0.98	0.99	0.99

*JAC: Jacareí-SP; SUL: Capão Bonito-SP; TLA: Três Lagoas-MS; TLS: Três Lagoas-MS.

The heritability estimates (h_q^2) can also confirm the existence of variability among polyploid clones. The lower limit of the estimate was positive in all cases, which indicates that the possibility of these limits being null is slight. It is important to stress that the results for DBH and MAI traits are very similar. However, the genetic variation was more accentuated among polyploids B, which exhibited h^2 above 0.8 for DBH, considering all sites. The estimates for MAI were also of high magnitude, above 0.69 (Table 3).

Table 3 Estimates of heritability for DBH (cm) and MAI (m^3 ha^{-1} $year^{-1}$) traits of polyploid eucalypt A and B (PA and PB), and its respective confidence intervals (UL: upper limit and LL: lower limit), at the age three in four sites.

SV	Site*	DBH			MAI		
		LL	h^2	UL	LL	h^2	UL
PA	JAC	0.13	0.42	0.64	0.17	0.45	0.66
	SUL	0.59	0.71	0.83	0.44	0.63	0.77
	TLA	0.45	0.63	0.78	0.43	0.62	0.77
	TLS	0.10	0.39	0.63	0.18	0.45	0.67
PB	JAC	0.69	0.80	0.88	0.53	0.69	0.81
	SUL	0.87	0.91	0.95	0.87	0.92	0.95
	TLA	0.82	0.88	0.93	0.80	0.86	0.92
	TLS	0.74	0.88	0.89	0.79	0.86	0.91

*JAC: Jacareí-SP; SUL: Capão Bonito-SP; TLA: Três Lagoas-MS; TLS: Três Lagoas-MS.

The joint analysis results for eucalypt DBH and MAI traits, considering the experiments conducted in all four sites, from 2015 to 2018, demonstrate the existence of variation among clones ($P \leq 0.0001$) (Table 4), as already indicated by the individual analyses. The clones x environments interaction was also significant ($P \leq 0.0001$), which suggests that the behavior of the clones was not coincident in the different sites. Nonetheless, the sum of squares of the interaction explained only 28% of the variation among clones for both traits (Table 4).

Table 4 Summary of the joint analysis of DBH (cm) and MAI (m^3 ha^{-1} $year^{-1}$) traits using BLUP means.

SV	DF	DBH	MAI
		MS	MS
Clone (C)	99	19.20^{***}	1734.40^{***}
Site (L)	3	89.57^{***}	3199.50^{***}
C x L	295	1.73^{***}	174.10^{***}
Error	4717	3.12	213.67

***: significant ($P \leq 0.0001$) in the F-test.

The decomposition of the clones x environments interaction was predominantly simple, i.e. the relative performance of clones was very similar among the evaluated sites. This is shown by the correlation estimates (*r*), which were high in most cases (above 0.69) (Table 5). In this scenario, it can be inferred that although present, the interaction did not expressively alter the ranking of clones in different sites.

Table 5 Estimates of correlation coefficient (*r*) and decomposition of the C x E interaction into simple and complex parts for DBH (cm) and MAI (m^3 ha^{-1} $year^{-1}$) traits of three-year-old eucalypt clones.

Sites*	DBH			MAI		
	r	**Simple Part (%)**	**Complex Part (%)**	***r***	**Simple Part (%)**	**Complex Part (%)**
1 x 2	0.82	33.73	66.27	0.83	44.04	55.96
1 x 3	0.83	53.60	46.40	0.87	68.25	31.75
1 x 4	0.82	41.78	58.22	0.85	46.79	53.21
2 x 3	0.69	55.65	44.35	0.75	77.82	22.18
2 x 4	0.70	48.97	51.03	0.75	69.39	30.61
3 x 4	0.97	37.78	62.22	0.98	55.53	44.47

*Sites - 1: JAC, 2: SUL, 3: TLA e 4: TLS (JAC: Jacareí-SP; SUL: Capão Bonito-SP; TLA: Três Lagoas-MS; TLS: Três Lagoas-MS).

The mean of the controls was consistently higher than the mean of polyploids A and B, which corroborates the ANOVA results (Table 3, Appendix). Nevertheless, the polyploids had better yield than the diploids in population A in the SUL experiment. In all the other cases, the mean of the controls was higher than the mean of polyploids, especially in the Três Lagoas' experiments (TLA and TLS). These can be verified by comparing the performance of diploids and polyploids in each site and the overall mean of all sites (Table 3, Appendix).

The mean performance of each polyploid clone from populations A and B, considering the mean of the four sites, is shown in Figures 2 and 3. Although the mean of polyploids A was lower than their diploid, some polyploids, such as clones 22 and 38, displayed mean performances similar to their diploid. Differences among polyploids B were also observed, confirming the ANOVA results. Regarding population B, the mean performance of polyploids was not close to the diploid in any case.

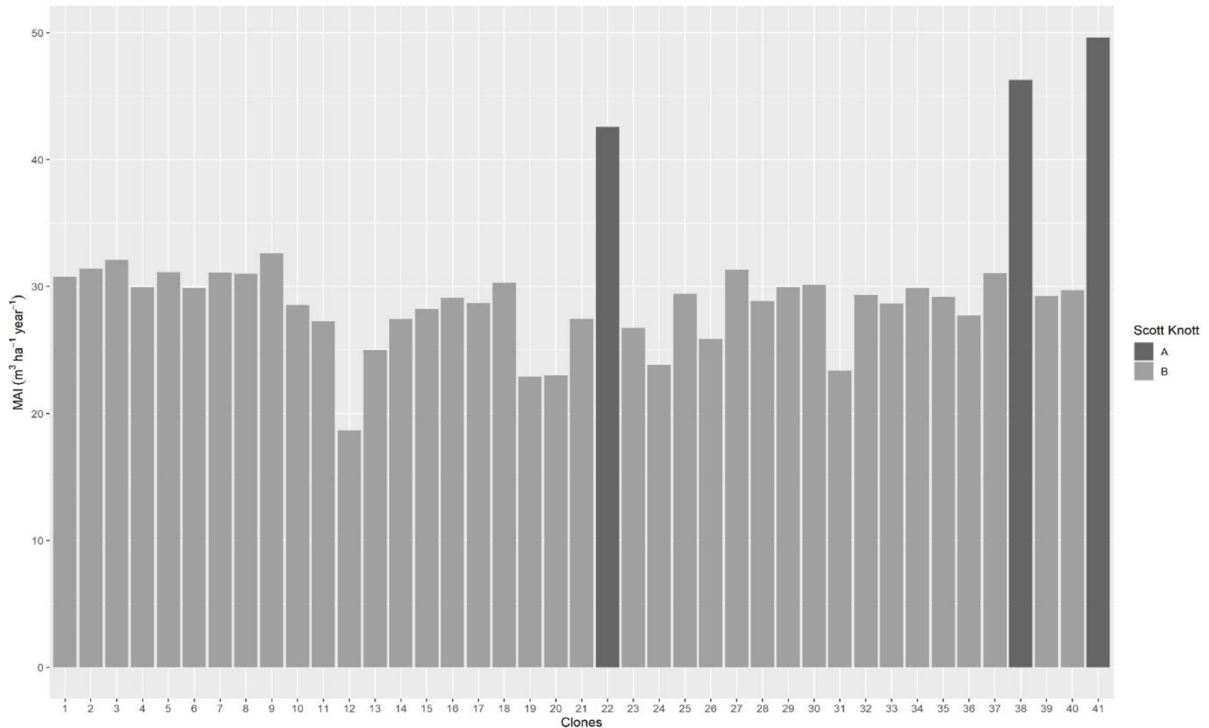

Figure 2 Estimates of mean MAI (m^3 ha^{-1} year^{-1}) of polyploids A (1 – 40) and diploid A (41) eucalypt clones (1 – 40 and 41 are the numbers of the clones in the clonal test). Average data from four sites at year three. Means followed by the same letter belong to same group according to the *Scott Knott* test (1974), at the probability of 5%.

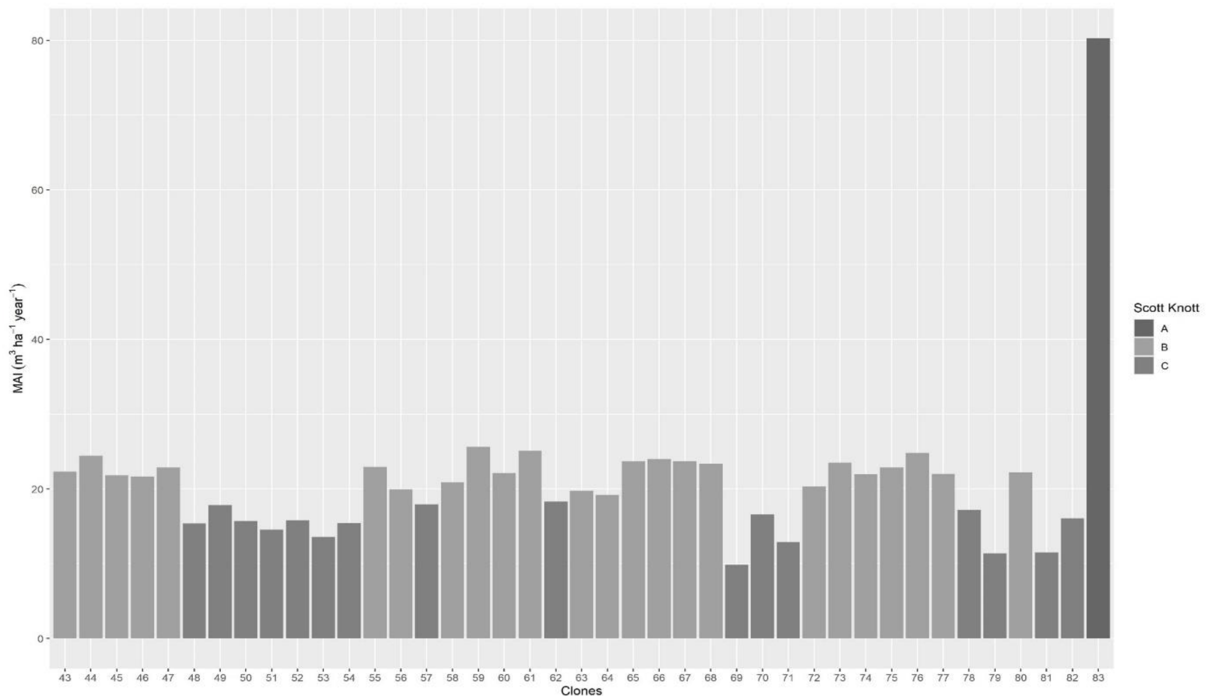

Figure 3 Estimates of mean MAI (m^3 ha^{-1} year^{-1}) of polyploids B (43 – 82) and diploid B (83) eucalypt clones (43 – 82 and 83 are the numbers of the clones in the clonal test). Average data from four sites at year three. Means followed by the same letter belong to same group according to the *Scott Knott* test (1974), at the probability of 5%.

The adaptability and stability of the evaluated polyploid and diploid clones are shown in Figures 4, 5, and 6 and Table 4 of the appendix. The adaptability is assessed through the grand mean of clones. The diploid A can be considered adapted compared to the other treatments because, apart from site SUL, its performance was above average. The polyploids 22 and 38 also stood out, with performances above the site mean. On the other hand, the polyploids 53, 81, and 82 displayed below average performance in all cases. The stability is indicated by the coefficient of variation (CV) of the variable Z in the different sites (Figure 5); the lower the CV, the more stable is the clone. Clones 7 and 9 were the most stable, however associated with lower adaptation (lower means).

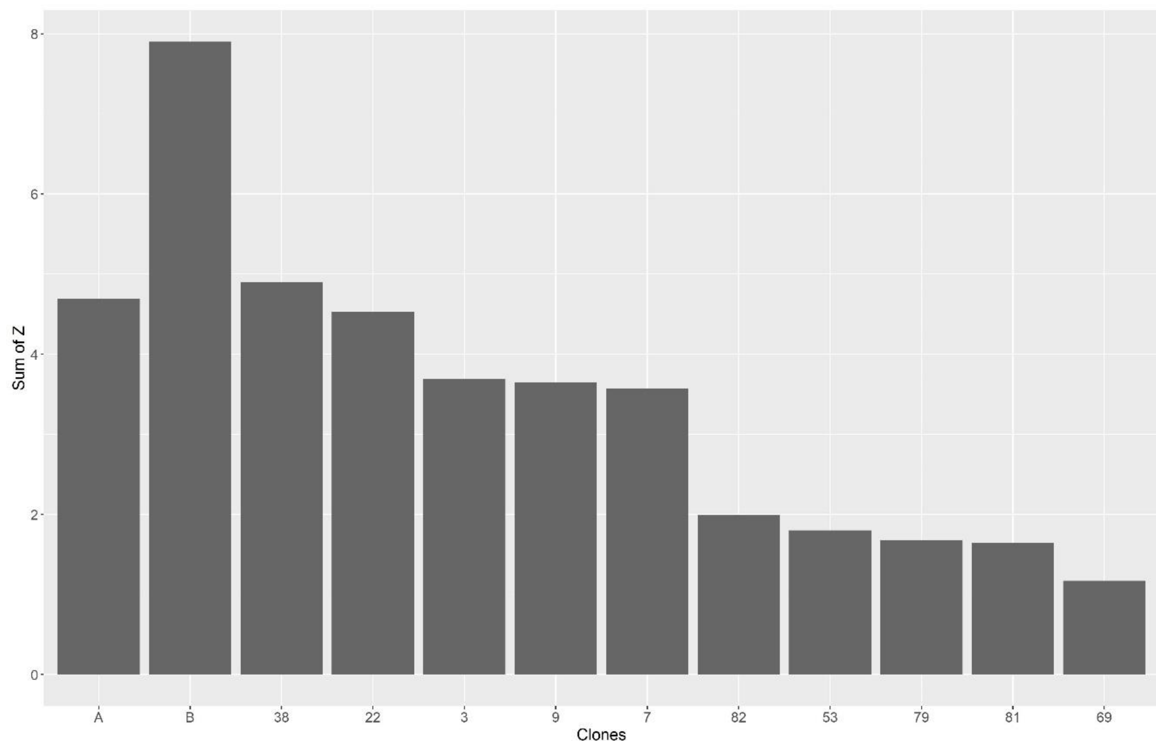

Figure 4 Sum of Z (standardized variable of MAI trait) of diploid eucalypt clones A, diploid B, polyploids A (3, 7, 9, 22, and 38) and polyploids B (53, 69, 79, 81, and 82).

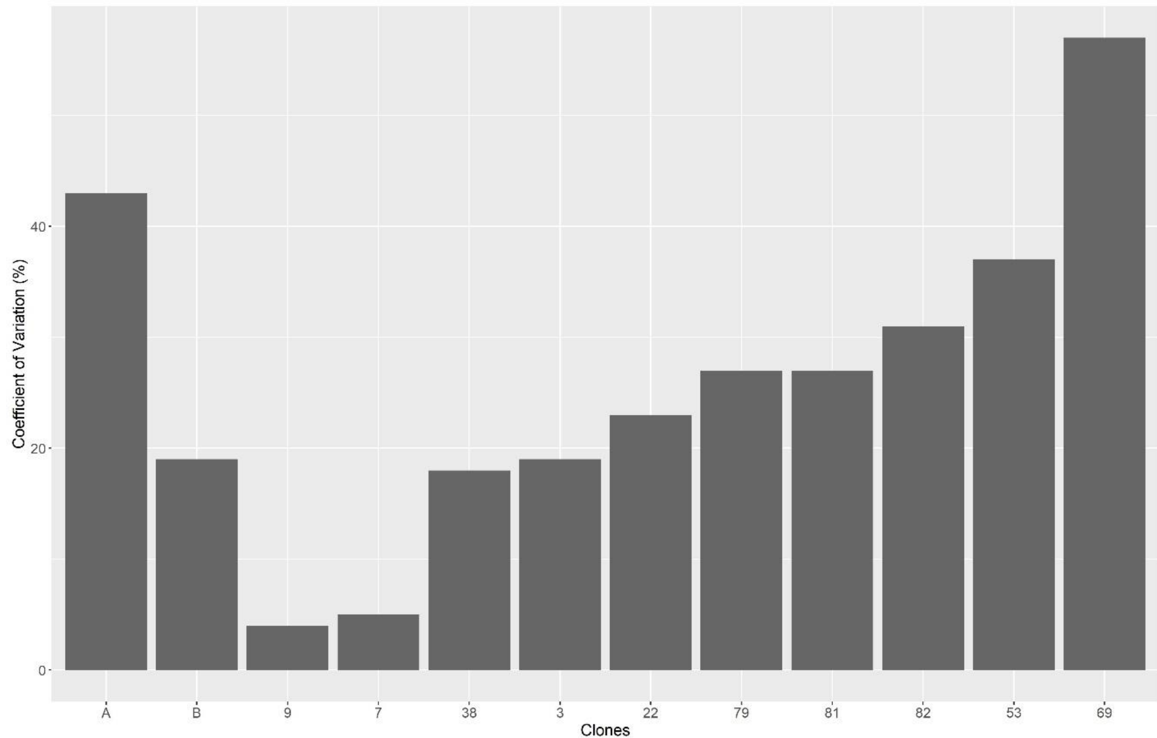

Figure 5 Coefficient of variation (%) of Z estimates of diploid eucalypt clones A, diploid B, polyploids A (3, 7, 9, 22, and 38) and polyploids B (53, 69, 79, 81, and 82).

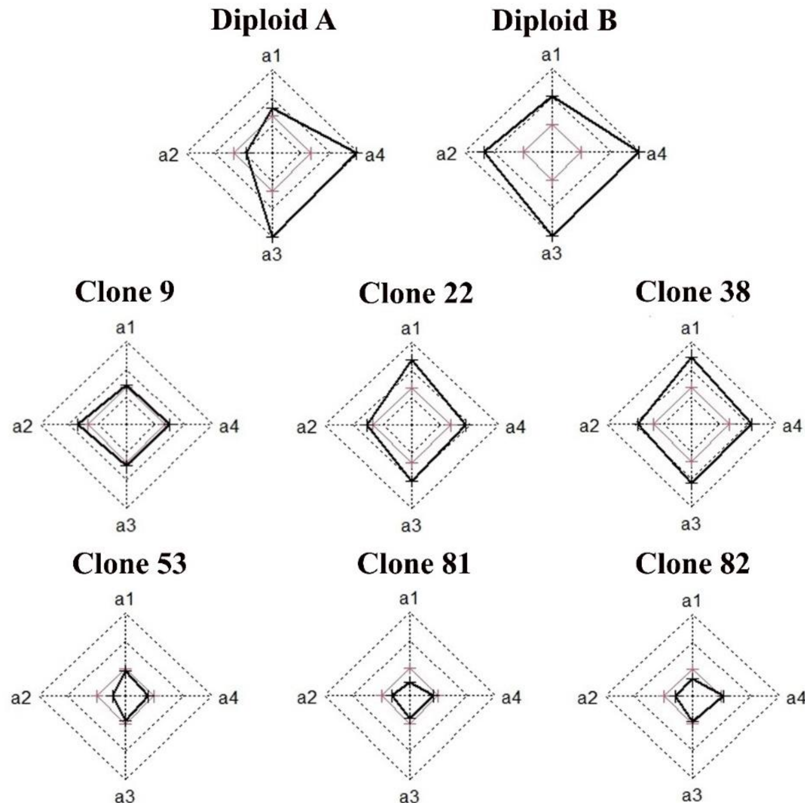

Figure 6 Graphic representation of MAI of polyploid eucalypt clones derived from diploids A and B, using the methodology proposed by Nunes et al. (2005). Red lines represent the overall mean of the 82 evaluated clones and black lines represent the performance of the clone in the four sites (a1: JAC, a2: SUL, a3: TLA and a4: TLS).

DISCUSSION

The environments where the experiments were conducted are representative of a significant portion of the areas where *Eucalyptus* is cultivated in Brazil. Eucalypt is grown under various conditions, including high altitude areas such as Capão Bonito and Jacareí-SP, and Atlantic Forest regions with good soil fertility, although low temperature events can reduce tree growth in specific periods. This reduced growth was observed in the SUL experiment, in 2016, during a period of low temperature averages in July, with the occurrence of frost. The mean temperature at the São Paulo locations in the mentioned period was 15ºC, while in Mato Grosso do Sul the mean temperature stayed above 20ºC (INMET, 2021). Eucalypt can also be grown in regions with higher temperatures and periods of accentuated water deficit, such as Três Lagoas-MS. Overall, the experimental sites displayed considerable variation in terms of altitude, geographic position, and climate conditions (Table 1).

Two alternatives were considered for the data analysis, a procedure similar to the one used by Bueno Filho and Vencovsky (2000). In the first alternative, we used a mixed model approach. In the second, we carried out the analysis of variance according to the least squares method (LSM), aiming to facilitate the decomposition of the source of variation treatments. It should be noted that the imbalance of the experiments was relatively small. This contributed to the correlation between the estimated LSM and BLUP means was high, above 0.98, indicating that the two procedures were coincident. In this context, both strategies generate similar results (Bernardo, 2020).

All the accuracy estimates (*rgg*) exhibited high values (Table 2), which demonstrates high experimental precision (Resende; Duarte, 2007). In general, when using a single tree plot, accuracy is usually high due to a large number of repetitions (Santos et al., 2021). In this context, an expressive difference was observed among treatments ($P \leq 0.01$) (Tables 1 and 2, Appendix), which means that the aim of the present study was successfully achieved, as significant differences were detected among genotypes, environments and for the genotype by environment interaction. Thus, the possibility of discriminating clones in terms of adaptability and stability was verified.

As mentioned, the DBH and MAI results were very similar. DBH and tree height are measured to estimate wood volume, and these traits are normally highly correlated (Machado; Figueiredo Filho, 2009), as observed in the present study. In this sense, the MAI results reflect the measures of DBH and height since the wood volume was used to estimate MAI. Therefore, we decided to discuss the results based on the MAI values, avoid redundant information, and

extract more practical conclusions, considering the direct applications desired by the company, which involves wood yield by area, annually.

The occurrence of G x E interaction in *Eucalyptus* progenies or clones is recurrent in Brazil (Souza, et al., 2017; Santos, et al., 2015; Rocha et al., 2005; Nunes et al., 2002). However, there is no report of studies accounting for G x E interaction in polyploids, and there are numerous methodologies that could be applied in studies on adaptability and stability (Eeuwijk et al., 2016). In our study, due to the small number of environments/sites evaluated, we used two strategies: decomposition of the interaction into simple and complex parts (Robertson, 1959) and the graphic method, according to Nunes et al. (2005).

As the correlation estimates (r) among sites, two by two, was relatively high in most cases, we inferred that the interaction was relatively simple (Table 5). This means that the relative performance of the clones was very similar among the evaluated locations, that is, it did not significantly change the ranking of the clones in the different environments.

The second method used to further investigate the interaction showed that there are differences among clones for adaptability and stability, as indicated, respectively, by the sums of the Z variables standardized by site and the coefficient of variation (CV) of the Z estimates among sites. The smaller the fluctuation in clone performance among sites, the smaller the CV. Nonetheless, in most cases, the stability is biological (Lin et al., 1986), which means that the CV estimate correlates negatively with the mean. This can be seen in the performance of polyploids 7 and 9, which are the most stable clones, but still, exhibit low performance. The polyploids 22 and 38 are also noteworthy, as they displayed the highest values for the sum of Z, and low CV estimates. This implies that their performance follows the improvement of the site mean, which can be characterized as agronomic stability, a desirable trait. Thus, in general, the stability among the evaluated clones is variable and is not always related to greater adaptation (Figures 4 and 5, and Table 4 from the Appendix).

The main objective of this study, however, was to verify if polyploid clones originated from the same diploid clone differ in adaptability and stability among themselves and in relation to their diploid of origin. We also aimed to verify if the performance of the polyploid clones was affected by their diploid origin.

The polyploid clones A were more adapted than polyploids B, as indicated by higher means (Figures 2 and 3). However, they present similar or inferior performance to their diploids, while diploid B performed much better than all polyploid clones. As mentioned, diploid clones A and B are considered adapted and stable, with performances above the grand mean in all sites. Some polyploid clones, such as 22 and 38, behaved similarly. On the other

hand, the polyploid clones 53, 81, and 82, of B origin, displayed below average performances, usually with high performance variation among locations (Figure 6). These results demonstrate that a high performing diploid will not necessarily produce high performing polyploid.

Considering these findings, the greatest challenge is to assess the viability of using polyploids in eucalypt breeding, and if viable, one should determine the best strategies to move forward. One positive outcome of using polyploidy was shown in a study with *E. grandis x E. urophylla*, in which tetraploid plants exhibited fiber with a higher length than diploids (Souza et al., 2021). In this case, a new question arises: would the investment in polyploid induction programs be justified based only on fiber quality?

In general, the most widely known consequence of polyploidization is the *gigas* effect, which refers to the enlargement of plant organs due to the increased number of gene copies (Sattler et al., 2016). Based on this premise, it was expected that polyploid clones would have superior vegetative development than their diploid. During early developmental stages at the green house, polyploid plants outperformed the diploids in vegetative growth (Souza et al., 2021). However, although polyploids presented a larger leaf surface, their photosynthetic efficiency was lower than diploids (data not presented). This must have caused the diploids to improve their performances over time gradually, ultimately surpassing the performance of tetraploids (Figures 2 and 3).

What would be the causes for polyploids to have lower performances than their diploids? One possibility is that each species must have an optimum ploidy level, refined by thousands of years of evolution. Several examples support this statement. The commercial strawberry (*Fragaria* × *ananassa*), for example, is an octaploid (2n=8x=56), but there are 22 wild species of *Fragaria* with ploidy levels ranging from diploids (2n=2x=14) to decaploids (2n=10x=70) (Edger et al., 2019). Another example is cotton, in which 95% of the production consists of an allotetraploid species (*Gossypium hirsutum*) due to its wide adaptability and high yield potential, while the remaining 5% consists mostly of *G. barbadense*, a diploid with longer and finer fibers. The genus *Gossypium* includes approximately 45 diploid (2n=2x=26) and five tetraploid (2n=4x=52) species, all exhibiting disomic inheritance patterns (Guan et al., 2014). *Urochloa*, a genus frequently used in pastures, has multiple ploidy levels. The most economically relevant species include: *U. brizantha* (2n=2x=18, 2n=4x=36, 2n=5x=45 or 2n=6x=54), *U. decumbens* (2n=2x=18 or 2n=4x=36), *U. ruziziensis* (2n=2x=18) and *U. humidicola* (2n=6x=36; 2n=7x=42 and 2n=9x=54) (Paula et al., 2017; Correa et al., 2020; Boldrini et al., 2006).

There are 754 *Eucalyptus* species (Powo, 2019), and the majority was classified according to the typological concept (Myburg et al., 2003), considering that in most cases, species can cross and produce viable and fertile progenies. Recently, some species were separated from the *Eucalyptus* to form the new genus *Corymbia*. Genomic analyses demonstrated that *E. grandis* has approximately twice the amount of DNA (640 Mb) than the *Corymbia* species *C. citriodora* subsp. *variegata* (370 Mb) and *C. torelliana* (390 Mb) (Butler et al., 2017). Further studies hypothesized *Eucalyptus* is a paleopolyploid genus, which is hinted by the same chromosome number in *Corymbia* and *Eucalyptus* (2n=2x=22), despite the difference in genome size (Butler et al., 2017). The eucalypt genome was shaped by a high rate of gene duplication (Myburg et al, 2014) and, therefore, it was characterized as a paleopolyploid, i.e. individuals who behave like diploids in terms of chromosome and genetic inheritance, but have been originated from past cycles of chromosome duplication. Considering this scenario, new questions arise: Would the polyploidization effect on a paleopolyploid be similar in situations where this phenomenon did not occur? Would there be an ideal ploidy level for *Eucalyptus*?

For breeding purposes, triploid plants (2n=3x=33) seem to be ideal because seed production is interrupted and therefore, part of its physiological resources could be redirected to vegetative development. Triploids can be more vigorous than diploids and importantly are frequently of low fertility or even sterile (Guo et al., 2017). However, trials to assess the growth, development and fiber quality of triploid eucalypt are still at preliminary stages.

A surprising finding from our analyses was the existence of expressive genetic variation among polyploids derived from a single clone. What would be the possible hypotheses to explain such variation? One possibility is the occurrence of mixoploidy in these clones (unpublished data) since it is a common phenomenon in artificial polyploidization protocols (Julião et al., 2020). Mixoploidy refers to the regeneration of plants, organs or tissues which cells differ in chromosome numbers or ploidy levels (Pereira et al., 2012; Pereira et al., 2017; Moura et al., 2020). For a successful duplication, the polyploidization agent must act on dividing cells at the end of phase S of the interphase and before cytokinesis. Therefore, even if the same polyploidization procedure is applied, mixoploidy occurs because the target tissue contains cells at different cell cycle stages (Pereira et al., 2012). Under these circumstances, the variations observed among polyploidized clones are likely due to different ratios of diploid and polyploid cells in their constitution. It is noteworthy that the polyploidization induction protocol used in this study was the same for the clones evaluated.

Another aspect that should be considered is the elimination of chromosomes or DNA fragments post-polyploidization. Such events have been observed in other artificially polyploidized species (Reis et al., 2016; Julião et al., 2020) and may impact on the phenotype. According to Julião et al. (2020) and Tayalé & Parisod (2013), in addition to numerical variation, structural rearrangements are frequently reported in newly formed polyploids, and such alterations can lead to new allele polymorphisms between individuals. In a study carried out by Buggs et al. (2008), triploids exhibited the highest rates of polymorphism and new alleles compared to the diploid mother plant, results that, according to the authors, can be explained by the DNA and genomic shock.

The unanswered question is what would be the best condition to promote larger vegetative development, the smaller number of polyploid cells and the larger number of diploid cells, or the opposite? Do diploid meristematic cells have any advantage over polyploid cells during mitosis? If so, would this contribute to a larger production of diploid cells?

Our study found that the diploid clone with the best MAI performance did not produce polyploid clones with similar performance. We also found that the polyploids differ among themselves, which indicates that a larger number of polyploids should be obtained before selection. In this context, what would be the best strategy to select diploid clones for polyploidization? We can also question if it is preferable to produce few polyploids from several diploid clones or generate more polyploid events from a single diploid. In some species, polyploids are produced from newly germinated seeds. Would this be the best option for obtaining a larger number of polyploids in *Eucalyptus*? If so, one could obtain hybrid seeds of two or more well-adapted clones, i.e. full-sib families, duplicate them and evaluate the polyploids considering the structure of the progenies. This would be an excellent opportunity to generate more variability and improve the selection of polyploid clones.

Polyploid research in *Eucalyptus* still presents several unanswered questions that need to be addressed in further studies. Nonetheless, current results suggest that polyploidy is a promising approach for eucalypt plantations.

CONCLUSIONS

Polyploids differed in adaptability and stability, and in most cases, they exhibit lower performances than diploids. The relative behavior of polyploid clones does not depend on the performance of the diploid that originated it since the most adapted polyploid clones were originated from the diploid clone with the lowest performance. Finally, our results showed that the use of polyploidy in the *Eucalyptus* breeding is still an open field for research and new findings.

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APPENDIX

Table 1 Summary of the analyses of variance for DBH (cm) trait of three-year-old eucalypt at the four sites (JAC, SUL, TLA e TLS). Decomposition of the effect of clones into diploids and polyploids A (DA and PA), diploids and polyploids B (DB and PB) and controls (C).

SV	DF	MS			
		JAC	SUL	TLA	TLS
Clones	99	3.16^{**}	1.96^{**}	10.85^{**}	8.64^{**}
Controls	17	2.61^{**}	2.80^{**}	3.83^{**}	5.27^{**}
PA	40	0.53^{*}	0.51^{**}	1.03^{**}	0.58^{**}
DA vs. PA	1	0.38^{ns}	2.26^{**}	18.88^{**}	9.17^{**}
Between PA	39	0.54^{*}	0.46^{**}	0.57^{*}	0.36^{ns}
PB	40	1.51^{**}	1.65^{**}	3.17^{**}	2.0^{**}
DB vs. PB	1	7.32^{**}	17.88^{**}	106.90^{**}	74.12^{**}
Between PB	39	1.36^{**}	1.23^{**}	0.51^{ns}	1.07^{**}
PA vs. PB	1	24.81^{**}	2.20^{**}	138.64^{**}	119.66^{**}
Rep	19	6.78^{**}	12.43^{**}	19.70^{**}	13.52^{**}
Error	●	0.31	0.14	0.38	0.35

**, * e ^{ns}: significant ($P \leq 0.01$), significant ($P \leq 0.05$) and not significant ($P > 0.05$) in the F-test, respectively.
●: degrees of freedom for JAC, SUL, TLA e TLS (1313, 1238, 968 e 1198, respectively).

Table 2 Summary of the analyses of variance for MAI (m^3 ha^{-1} $year^{-1}$) trait of three-year-old eucalypt at the four sites (JAC, SUL, TLA e TLS). Decomposition of the effect of clones into diploids and polyploids A (DA and PA), diploids and polyploids B (DB and PB) and controls (C).

SV	DF	MS			
		JAC	SUL	TLA	TLS
Clones	99	269.59**	96.88**	1163.53**	744.85**
Controls	17	241.68**	141.41**	682.84**	635.42**
PA	40	41.46*	14.65*	100.73*	44.36*
DA vs. PA	1	25.47ns	25.84*	2450.95**	962.00**
Between PA	39	41.87*	14.37*	40.47ns	20.83ns
PB	40	73.05**	64.39**	282.06**	170.46**
DB vs. PB	1	724.07**	1083.82**	10856.52**	6178.17**
Between PB	39	56.35*	38.25**	10.92ns	16.41ns
PA vs. PB	1	1084.29**	1077.50**	6794.30**	4108.96**
Rep	19	638.17**	422.04**	1187.90**	872.44**
Error	●	22.97	5.46	38.53	24.19

**, * e ^{ns}: significant ($P \leq 0.01$), significant ($P \leq 0.05$) and not significant ($P > 0.05$) in the F-test, respectively.
●: degrees of freedom for JAC, SUL, TLA e TLS (1313, 1238, 968 e 1198, respectively).

Table 3 Estimates of means of DBH (cm) and MAI (m^3 ha^{-1} $year^{-1}$) traits of three-year-old eucalypt clones at four sites (JAC, SUL, TLA e TLS) and performance of diploids in relation to polyploids in percentage (%), by site and site mean.

Clone	JAC	%	SUL	%	TLA	%	TLS	%	Mean	%
DBH										
Diploid A	12.29	5.1	7.84	-16.2	14.58	30.2	13.19	23.3	11.97	13.7
Polyploids A	11.66		9.36		10.18		10.12		10.33	
A	11.97		8.60		12.38		11.65		11.15	
Diploid B	13.25	20.7	13.17	32.5	17.61	58.0	16.28	53.5	15.08	43.0
Polyploids B	10.51		8.89		7.40		7.57		8.59	
B	11.88		11.03		12.50		11.93		11.84	
Controls	14.43	23.2	11.15	18.1	15.85	44.5	14.83	40.4	14.06	32.7
Polyploids	11.09		9.13		8.79		8.84		9.46	
MAI										
Diploid A	41.90	12.2	17.42	-22.8	78.48	63.9	60.64	51.8	49.61	41.1
Polyploids A	36.79		22.57		28.35		29.24		29.24	
A	39.35		19.99		53.41		44.94		39.42	
Diploid B	56.22	48.5	56.03	59.5	115.32	89.2	93.49	85.1	80.27	75.7
Polyploids B	28.98		22.70		12.44		13.91		19.51	
B	42.60		39.37		63.88		53.70		49.89	
Controls	66.64	50.7	39.44	42.6	97.35	79.0	81.27	73.5	71.17	65.8
Polyploids	32.88		22.63		20.40		21.57		24.37	

Table 4 Estimates of coefficients of variation (%) and sum of Z of eucalypt clones from population A (polyploids: 1-40, diploid: 41) and from population B (polyploids: 43-82, diploid: 83) at four sites.

Population A			Population B		
Clone	**CV (%)**	**∑ Z**	**Clone**	**CV (%)**	**∑ Z**
1	7	3.49	43	21	2.87
2	7	3.56	44	27	3.14
3	19	3.69	45	23	2.77
4	8	3.41	46	22	2.83
5	7	3.53	47	10	2.85
6	13	3.41	48	25	1.94
7	5	3.57	49	17	1.99
8	7	3.47	50	24	1.99
9	4	3.65	51	18	1.95
10	6	3.29	52	14	2.12
11	12	3.09	53	37	1.8
12	17	2.28	54	15	2.01
13	18	2.83	55	26	2.99
14	12	3.13	56	35	2.66
15	11	3.2	57	40	2.3
16	8	3.29	58	23	2.71
17	9	3.24	59	22	3.23
18	4	3.47	60	20	2.82
19	40	2.55	61	18	3.12
20	41	2.52	62	34	2.44
21	15	3.23	63	20	2.58
22	23	4.53	64	19	2.5
23	24	3	65	16	2.98
24	21	2.73	66	24	3.07
25	4	3.37	67	21	3.02
26	9	2.98	68	27	2.99
27	8	3.48	69	57	1.17
28	9	3.27	70	13	2.12
29	15	3.4	71	22	1.84
30	9	3.37	72	30	2.75
31	11	2.77	73	23	2.99
32	7	3.37	74	12	2.75
33	4	3.31	75	23	2.97
34	6	3.49	76	22	3.15
35	8	3.35	77	32	2.85
36	10	3.16	78	10	2.31
37	7	3.47	79	27	1.68
38	18	4.9	80	21	2.87
39	3	3.37	81	27	1.64
40	3	3.4	82	31	1.99
41	43	4.69	83	19	7.9