ALEX JUNIOR DA SILVA

CHROMOSOME SET DOUBLING AND DNA METHYLATION AS TRIGGERS OF PHENOTYPIC VARIABILITY IN *Eucalyptus* sp.

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Doctor Scientiae*.

Orientador: Aluízio Borém de Oliveira

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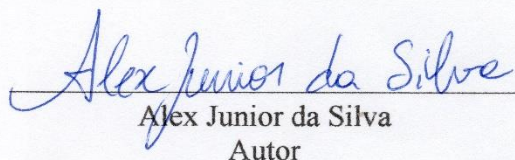

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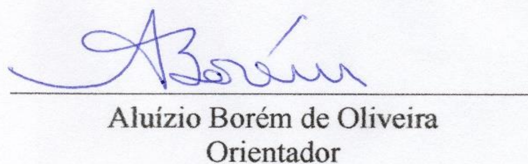

Aluízio Borém de Oliveira
Orientador

Aos meus pais Erci e Janeti. À minha irmã Karen. À minha amiga Cassia. Ao meu professor de Genética Nilton César Mantovani, por onde tudo começou.

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“Só um sentido de invenção e uma necessidade intensa de criar levam o homem a revoltar-se, a descobrir e a descobrir-se com lucidez”.

(Pablo Picasso)

RESUMO

SILVA, Alex Junior da, D.Sc., Universidade Federal de Viçosa, dezembro de 2020. **Duplicação do conjunto cromossômico e metilação de DNA como desencadeadores da variabilidade fenotípica em *Eucalyptus* sp.** Orientador: Aluízio Borém de Oliveira.

Mudanças genômicas como a duplicação do conjunto cromossômico e padrões de metilação do DNA, têm um significado profundo nos aspectos ecológicos e evolutivos, moldando as plantas em seus diferentes ambientes e condições adversas. Nesse contexto, mudanças induzidas no genoma por duplicação ou manipulação nos padrões de metilação do DNA podem criar fenótipos vantajosos, herdáveis, estáveis e, portanto, passivos de seleção em programas de melhoramento. Nesse sentido, avaliamos o efeito da duplicação do conjunto cromossômico em árvores de *Eucalyptus benthamii* no contexto do metabolismo secundário e da desmetilação do DNA global em sementes germinadas de *Eucalyptus urograndis,* durante a organogênese *in vitro*. Árvores autotetraploides de *E. benthamii* produziram 1,8-cineol, limoneno, α-terpineol e acetato de α-terpinila que, em contraste, não foram identificados em árvores diploides, e o rendimento dos óleos essenciais não foi correlacionado com o nível de ploidia. Os óleos essenciais de diploides têm duas vezes mais efeito larvicida em larvas de *Aedes aegyptii* do que os autotetraploides. Os óleos essenciais de ambos os níveis de ploidia não apresentaram efeito citogenotóxico em raízes meristemáticas de *Lactuca sativa*. Além da alteração no nível de ploidia cromossômica, mudanças epigenéticas nos padrões globais de metilação de DNA podem promover fenótipos estáveis de interesse florestal. Em plântulas de *E. urograndis*, o uso de 5-azacitidina reduziu os níveis de 5-metil citosina em todas as partes da planta (raízes, hipocótilos, cotilédones e folhas). Tratamentos com este desmetilante promoveram um aumento de 65% na formação de raízes, sem auxílio de fitohormônios. A desmetilação subsequente do DNA por organogênese promoveu uma redução de 50% na metilação global do DNA de plantas regeneradas. A duplicação do conjunto cromossômico e a desmetilação do DNA são ferramentas poderosas para modificar a diversidade fenotípica em curto prazo, e em longo prazo essas alterações podem ser exploradas visando características desejáveis no melhoramento florestal.

Palavras-chave: Poliploidia. Epigenética. Melhoramento florestal. Biotecnologia. Eucaliptos.

ABSTRACT

SILVA, Alex Junior da, D.Sc., Universidade Federal de Viçosa, December, 2020. **Chromosome set doubling and DNA methylation as triggers of phenotypic variability in *Eucalyptus* sp.** Advisor: Aluízio Borém de Oliveira.

Genomic changes as chromosome set doubling and DNA methylation patterns have a profound significance in ecological and evolutionary aspects, shaping plants to different environments and adverse conditions. Within this context, induced changes in whole genome by duplication or manipulation of DNA methylation patters, could create advantageous phenotypes, inheritable and stable, and therefore, passive of selection in breeding programs. In this sense, we evaluated the effect of chromosome set doubling in *Eucalyptus benthamii* trees in the context of secondary metabolism, and whole DNA demethylation in germinated seeds of *Eucalyptus urograndis* during *in vitro* organogenesis. Autotetraploid trees of *E. benthamii* produced 1,8-cineole, limonene, α-terpineol, and α-terpynil acetate which in contrast were absent in diploids, and yielding of essential oils were not correlated to the ploidy level. Diploid essential oils have twice more larvicidal effect against *Aedes aegyptii* larvae than autotetraploids. Essential oils from both ploidy levels had no cytogenotoxic effect in meristematic roots of *Lactuca sativa*. Besides the ploidy level alterations, epigenetic changes in the global DNA methylation levels may promote stable phenotypes of forest interest. In *E. urograndis* plantlets, using 5-Azacytidine it was possible to reduce 5-methyl cytosine levels in whole plant (roots, hypocotyls, cotyledons and leaves). Treatments with this demethylating agent promoted an increase of 65% in rooting formation without plant growth regulators. Subsequent DNA demethylation through organogenesis promoted a reduction of 50% in global DNA methylation of regenerated plants. Chromosome set doubling and DNA demethylation are powerful tools to modify phenotypic diversity in short-term, and in long-term these changes can be more explored aiming desirable traits in forest breeding.

Keywords: Polyploidy. Epigenetic. Forest breeding. Biotechnology. Eucalypts.

SUMÁRIO

1. GENERAL INTRODUCTION 12
2. REFERENCES 15
3. RESEARCH PAPER 1: Short-term changes related to autotetraploidy in essential oil composition of Eucalyptus benthamii Maiden & Cambage and its applications in different bioassays 18
3.1 Abstract 19
3.2 Introduction 20
3.3 Materials and methods 23
Plant material and ploidy level stability 23
EO extraction and yield 24
EO chromatography 25
Larvicidal activity 26
Phytotoxicity bioassay 27
Cytogenotoxicity bioassays 28
EO extraction, profiling, and germplasm characterization 29
3.5 Discussion 38
3.6 Conclusions 43
3.7 References 44
4. RESEARCH PAPER 2: Inducing DNA demethylation in *Eucalyptus urophylla* × *Eucalyptus grandis*: new epigenetic germplasm? 53
4.1 Abstract 54
4.2 Introduction 55
4.3 Materials and Methods 57
Plant material and explant source for demethylation experiments 57
Germination and rooting under 5-AzaC 58
Callus induction, shooting and plantlet recovery 58
Global DNA methylation levels 59
4.4 Results 62
Germination and rooting under 5-AzaC 62
Subsequent DNA demethylation using 5-AzaC in organogenesis system.............. 64
4.5 Discussion 68
Effect of 5-AzaC on germination and potential applications in rooting 68

Subsequent DNA demethylation: adding an extra layer of complexity 69
4.6 Conclusions 71
4.7 References 72
5. OVERALL CONCLUSIONS 76

1. GENERAL INTRODUCTION

The increased demand for renewable resources, such as wood, pulp and paper, lead researchers to expand the horizons of options to accelerate breeding programs, specially concerning to forest tree species – that have a long juvenile period and long lifespan (Bhalerao et al., 2003). *Eucalyptus* is widely the most cultivated woody angiosperms around the world (Ladiges et al., 2003), and given to its rapid growth and fast adaptation in many countries, has been target of different approaches in plant breeding and biotechnology (Boerjan, 2005; Grattapaglia, 2008). Some of these efforts were developed recently given to the urgency of accelerating productivity in different scenarios, such as chromosome set doubling (CSD) (Silva et al., 2019), genomic wide association (Resende et al., 2017), genetic transformation (França Bettencourt et al., 2020) and epigenetic studies associated to environmental responses (Correia et al., 2016; Pereira et al., 2020).

CSD or polyploidy, is a natural phenomenon defined by the presence of more than two entire sets of chromosomes in a single nucleus, which almost all angiosperms has experienced this event (Soltis et al., 2009). In short term, new polyploids (neopolyploids) have rapid modifications in gene expression, dosage effect, new allelic combinations and even epigenetics patterns (Ramsey and Schemske, 2002). In long term, the extra copies of whole genome allow gene neo or subfunctionalization, or gene silencing due to adjustments of molecular mechanisms overtime (i.e. DNA methylation, chromatin remodeling and transposable elements) (Jackson and Chen, 2010; Spoelhof et al., 2017). These changes are able to increase phenotype diversity and plasticity, bringing novelty to plants.

In this sense, CSD has been applied artificially in single species (autopolyploids) or hybrid trees (allopolyploids). In *Platanus acerifolia* (Ait.) Willd. CSD was aimed to reduce

pollen and fruiting dropping in urban areas (Liu et al., 2007), while for *Acacia dealbata* Link. and *Acacia mangium* Willd. reduce seed production through triploids, diminishing the effects of invasiveness (Blakesley et al., 2002). For *Eucalyptus* sp., the first reports came from the last 10 years (Lin et al., 2010; Han et al., 2011; Silva et al., 2019; Fernando et al., 2019; da Silva Souza et al., 2020), although only da Silva Souza et al. (2020) has characterized fibers measurements, indicating an increase of 18% of fiber length in allotetraploid clones when compared to their diploid counterparts. Moreover, the impact of CSD regarding in the secondary metabolism are still needed to better understand the new network stablished in polyploid *Eucalyptus* trees.

Epigenetic mechanisms also contribute to regulate plant growth and development, as well as shaping the phenotype variability (Duarte-Aké et al., 2019). Epigenetic marks include DNA methylation and histone post-translational modification that are inherited through mitosis or meiosis with no DNA sequence alteration. Epigenetic regulate gene expression, chromatin structure, transposable elements and even DNA recombination (Fortes and Gallusci, 2017). The most studied epigenetic modification is DNA methylation, 5-methylcytosine, which is modulated by DNA methyltransferases add a methyl radical at the 5th carbon of a pyrimidine ring of a cytosine. These DNA methyltransferases maintain the marks into the new DNA strands during cell divisions (Us-Camas et al., 2014).

Since DNA methylation are involved in many process, epigenetic variants may represent a large scope for plant breeding (Latutrie et al., 2019), where the most benefits can be captured by species clonally propagated, such as *Eucalyptus.* Epigenetic variants with agronomical interest have been identified, like dwarf phenotypes rice (Chen and Zhou, 2013), anthocyanin production in apples (Telias et al., 2011) and sex determination in melon (Martin et al., 2009). Moreover, DNA methylation can be manipulated artificially using demethylating chemicals such as 5-Azacitydine. 5-Azacytidine inhibit DNA

methyltransferase activity by formation of a covalent complex, preventing DNA methylation formation into the newly synthetized DNA strands. This strategy has been used to explore different traits such as root improvement (Massoumi et al., 2017), smut and Imazapyr (herbicide) tolerance (Munsamy et al., 2013), or even to increase phenotypic variability such branches and flowering time (Bossdorf et al., 2010).

Considering the major role of CSD and DNA methylation shaping phenotypic variability in plants, we aimed to characterize the effect of CSD in the context of secondary metabolism of *E. benthamii*; and also promote a global DNA methylation in order to create an epigenetic germplasm of *E. urograndis.* From both studies, its was possible to diversify *Eucalyptus* materials, incorporating new germplasm for tree improvement. Furthermore, these materials make an *in vivo* experiment for further characterization of changes at genomic scale.

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3. RESEARCH PAPER 1: Short-term changes related to autotetraploidy in essential oil composition of Eucalyptus benthamii Maiden & Cambage and its applications in different bioassays

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Keywords: polyploidy, genotoxicity, phytotoxicity, allelopathy, secondary metabolism.

3.1 Abstract

Some forest trees have been polyploidized to improve their traits and to supply new germplasm for breeding programs. As trees have a long juvenile stage, the early characterization of the chromosome set doubling effects is crucial for previous selection. Thus, we aimed to characterize the chemical variability of essential oils from diploid and autotetraploid germplasm (autotetraploid A and B) of *Eucalyptus benthamii*, as well as to evaluate their larvicidal and allelopathic effects. Autotetraploid A showed a higher essential oil yield than diploid and autotetraploid B, which did not differ quantitatively. Aromadendrene, viridiflorol, and α-pinene were the major compounds in the diploid essential oil. In contrast, compounds such as 1,8-cineole, limonene, α-terpineol, and α-terpynil acetate were present in autotetraploids. Essential oils from the diploid at 50–200 ppm were twice as larvicidal than those from autotetraploids against *Aedes aegypti* larvae. Considering the phytotoxicity bioassays using *Lactuca sativa*, essential oils from both ploidy levels inhibited root growth at 1,500 and 3,000 ppm. Moreover, the essential oils inhibited shoot growth at all concentrations tested (187.5; 375; 750; 1,500; and 3,000 ppm). Autotetraploid A and B had the same effect on shoot growth as glyphosate. The essential oils had no cytogenotoxic effect on root meristematic cells of *L. sativa,* whereas phytotoxic potential was identified mainly in shoot growth. This work demonstrated a dramatic change in secondary metabolism (terpene composition) related to an increase in the ploidy level in *Eucalyptus* germplasm. In addition, we report the novelty of the chemical composition of essential oils among germplasm and their potential use as larvicidal and post-emergence weed control agents.

3.2 Introduction

Eucalyptus is an important genus among the cultivated woody angiosperms, and it includes more than 700 species of trees and shrubs (Ladiges et al., 2003). *Eucalyptus* belongs to the Myrtaceae Family, which is prolific in species that produce essential oils (EOs) (Goodger et al., 2018). From more than 300 different EOs of commercial value, at least 20 are found in the genera *Eucalyptus* and *Corymbia*, which possess species characterized by high EO production (Boland et al., 1991).

Eucalyptus EOs are volatile, odoriferous, hydrophobic liquids, soluble in alcohol, and appear pale yellow, brownish, greenish, or even colorless (Araujo et al., 2010). EOs are composed of hydrocarbons, oxygenated monoterpenes, sesquiterpenes, diterpenes, and aromatic and aliphatic compounds with low molecular weights (Barbosa et al., 2016). In *Eucalyptus,* EOs are composed by monoterpenes and sesquiterpenes, stored in secretory cells, the "oil glands," abundant in leaves (Naidoo et al., 2014). Terpene biosynthesis occurs through terpene synthase enzymes that are involved in two metabolic pathways: 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in the plastids and the mevalonic acid (MVA) pathway in the cytosol and peroxisomes (Chen et al., 2011). Terpene production is compartmented; monoterpenes, diterpenes, and tetraterpenes are produced mainly in plastids; sesquiterpenes and triterpenes are produced in the cytosol; and oxidized terpenes are produced in mitochondria and peroxisomes (Aharoni et al., 2005; Chen et al., 2011).

Exploration of *Eucalyptus* EOs dates from the 19th century in Australia (Davis, 2002). Since then, advances in silviculture have been optimized to maximize EO production, such as selection and crossing involving superior genotypes (Silva et al., 2006). Breeders were able to select important phenotypic features to improve EO yield and tree adaptation to different environments in some species, such as *Corymbia citriodora* (Hook.) K.D. Hill & L.A.S. Johnson, *Eucalyptus camaldulensis* Dehnh., *Eucalyptus radiata* D. C., *Eucalyptus*

globulus Labill., and *Eucalyptus staigeriana* F. Muell (Doran, 2002). Phytochemically, *Eucalyptus* EOs can have citronellal, citronellol, 1,8-cineole, α-pinene, α-terpineol, limonene, geranyl acetate, and linalool, among other major compounds (Barbosa et al., 2016). The chemical components as well as the yield of EOs may vary according to the environment. For example, *E. camaldulensis* from Argentina contains 1,8-cineole, p-cymene, β-phellandrene, and 0.38% EO yield, while in Brazil, the same species has 1,8-cineole, limonene, γ-terpinene, α-pinene, and 0.63% yield (Batista-Pereira et al., 2006; Lucia et al., 2009). Despite the characterization of EO from *E. benthamii* leaves (Döll-Boscardin et al., 2012), these trees are mostly planted for the production of cellulose, timber, or bioenergy. As such, leaves are left in the field as litter for biomass decomposition and have not yet been explored for EO extraction.

Aiming for germplasm diversity, synthetic autotetraploid and allotetraploid *Eucalyptus* have been produced to be incorporated in breeding programs and in the field (Lin et al., 2010; Silva et al., 2019). As the *Eucalyptus* nuclear genome potentially has 113 genes related to terpene synthesis (Chen et al., 2011), chromosome set doubling (CSD) procedures increase the gene copy and, consequently, increase the phenotypic diversity of EO production and composition (Bhuvaneswari et al., 2020). Regarding secondary metabolites, autotetraploids of *Citrus paradisi* Macf. and of *Citrus limon* (Linn.) Burm showed more 5.4% α-terpineol and 2% geranial, respectively, than their diploid relatives (Cameron and Scora, 1968). Moreover, the autotetraploid condition in *Cymbopogon* spp. led plants to produce 33%, 5.67%, and 4.73% more biomass, citral, and geraniol, than their diploid counterparts, respectively (Lavania et al. 2012). Nevertheless, the increase in ploidy levels does not always increase traits of interest; in autotetraploids of *Citrus reticulata* Blanco and *Poncirus trifoliata* (L.) Raf., α-caryophyllene and β-elemene contents were reduced (Tan et al., 2017).

Therefore, the gene copy increases alleles per locus, generating more overdominance than in diploid counterparts (Ramsey & Schemske, 2002). The response for this effect covers the "omics" (genomic, epigenomic, transcriptomic, and metabolomic), resulting in different yield and composition of secondary metabolites such as EO (Xing et al., 2011; Marfil et al., 2018). In spite of CSD could enhance EO production, a careful screening and selection still needed among the autopolyploid or allopolyploid germplasm, or both, based on chemical profiling (Lavania et al., 2012). In *Eucalyptus polybractea* R. T. Baker, in spite of the superior size of the oil glands, EO yield were lower in autoptetraploids than their diploids counterparts as well as growth rates and stomata density (Fernando et al., 2019). A recent study with 36 months *E. urograndis* allotetraploid clones revealed an increase of 18% of fiber length, and 13% decrease basic density compared to diploid trees, indicating their potential use in pulp industry (da Silva Souza et al., 2020).

EO synthesis plays a central role in plants, providing defense against biotic and abiotic stresses (Tholl, 2006). Due to their chemical variability and toxicity, EOs are biomolecules extensively used as pesticides to control insect pests, plant diseases, and weeds. Moreover, other applications include medicine and cosmetics production (Batish et al., 2006; Aragão et al., 2015).

Neotropical countries, such as Brazil, have suffered from dengue, zika, yellow fever, and chikungunya epidemics, transmitted by *Aedes aegypti* L. (Rasmussen et al., 2013). To control *A. aegypti*, health agencies frequently use pyrethroids as insecticides, which have larvicidal effect at concentrations as low as 1 ppm. However, this control method can promote insect resistance and affect non-target organisms (Braga et al., 2004; Estep et al., 2017). Thus, EOs can be used due to their chemical complexity and neurotoxic effects, inhibiting adult emergence and inducing larval death (Isman, 2000; Shaalan et al., 2005). In addition, EOs are widely available for extraction and are considered as a "green alternative"

because they are rapidly degraded in the environment. Most monoterpenes, such as α-terpineol, have a half-life of 30–40 h in the environment, making them a feasible alternative due to their rapid degradation and the consequent short-time exposure of non-target species (Misra & Pavlostathis, 1997).

Despite the advantages of using EOs as natural pesticides, bioassays should be performed to determine EO toxicity and potential effects to different model organisms. *Lactuca sativa* L., for example, is used in toxicity bioassays as a model plant to assess the potential use of EOs in weed control. Allelopathic effect of EOs are described by reducing seed germination, rate germination, as well as shoot and root growth of plantlets (de Assis Alves et al., 2018). Moreover, bioassays using *A. aegypti* larvae can determine EO toxicity and its potential application as a pesticide (Aragão et al., 2015; Mendes et al., 2017). Through slide preparations, the identification of aneugenic and clastogenic effects (such as chromatin condensation, micronuclei, and chromosome loss and alterations) is used as a parameter to determine the toxicity of EOs (Andrade et al., 2010; Palmieri et al., 2016). Moreover, flow cytometry analysis is a tool that rapidly measures cell populations treated with new drugs and substances (Jayat & Ratinaud, 1993); thus, flow cytometry could be used to verify the effect of EOs on cell proliferation.

In the present study, we described and compared the chemical composition of foliar EOs of one diploid and two synthetic autotetraploid *E. benthamii* (Silva et al., 2019) germplasm. Additionally, we tested the larvicidal and allelopathic effects of these EOs.

3.3 Materials and methods

Plant material and ploidy level stability

Seeds of *E. benthamii* from open-pollinated crosses were germinated, and a diploid germplasm (2n = 2× = 22 chromosomes) was obtained and multiplied. Solid autotetraploid plantlets (2C = 1.22 pg, 2n = 4× = 44 chromosomes) were generated from two diploid plantlets submitted to the CSD procedure (Silva et al., 2019). Considering that *Eucalyptus* is predominantly allogamous and the germplasm were generated from two distinct seeds of *E. benthamii,* the autotetraploids were denominated here as autotetraploid A and autotetraploid B. Each sample from every germplasm was ranked according to EO yield (%) and denominated as 1 (lower yield), 2 (intermediate yield), and 3 (higher yield). Diploid and autotetraploids were vegetative propagated and developed during 2016 and 2017 at the Laboratório de Citogenética e Citometria (Universidade Federal de Viçosa, MG, Brazil). The ploidy level stability was checked by flow cytometry during in vitro propagation (Silva et al., 2019).

After three months of in vitro multiplication, the plantlets were acclimatized and transferred to the Klabin S.A. Experimental Research Station, Paraná, Brazil (24°13′31″S, 50°32′44″O). The trees were cultivated for 1.5 years and spaced 3 × 2 m, until they reached 5-m high and 12.5-m circumference at breast height. In the field, a new ploidy assessment was performed to check the DNA ploidy level stability. Leaves from nine trees (three from each germplasm) were collected during Autumn (April/May 2019), in three different portions along the longitudinal axis: basal, medium, and top of the crown. For each sample, 500 g of leaves were packed in kraft paper. After 24 h, the leaves were air-dried for seven days at 25 ± 1°C, and weighed in an analytical balance to obtain the leaves' dry mass. All samples were stored at -20 °C until use (Mendes et al., 2018).

EO extraction and yield

Leaves (50 g) from each sample were added to a round-bottom distillation flask (1 L) containing 600 mL of dH_2O. The flasks were attached to a Clevenger apparatus and a condenser. Hydrodistillation was performed for 3 h, and the vapor of oils and water was condensed and collected into a 125 mL Erlenmeyer flask, as recommended by the Brazilian Phamacopoeia for volatile oils (Brasil, 2010; Barbosa et al., 2016; Filomeno et al., 2017). The hydrolate was subjected to liquid-liquid extraction using dichloromethane (3 × 10 mL). The organic phase was dried with anhydrous sodium sulfate, filtered, and concentrated under reduced pressure in a rotatory evaporator. The obtained EO was transferred to 2 mL microtubes protected from light and stored at -20 °C. The EO yield was measured considering the dried mass of the leaves divided by the extracted EO mass in m m^{-1} (Barbosa et al., 2016). The yield data were subjected to ANOVA and the Scott-Knott test at 5%, and a correlation analysis was performed between yield (%) and composition (%).

EO chromatography

The extracted EO of each sample was analyzed via gas chromatography with a flame ionization detector (GC-FID, Shimadzu GC-2010 Plus) and gas chromatography coupled to mass spectrometry (GC-MS, Shimadzu GCMS-QP2010 SE). EOs were removed from the vials in 1 µL of a solution consisting of 3% EOs dissolved in hexane. The analysis was conducted following the equipment conditions: Helium (He) as carrier gas in both detectors, with flow and linear speed of 2.80 mL min^{-1} and 50.8 cm sec^{-1} (GC-FID), and 1.98 mL min^{-1} and 50.9 cm sec^{-1} (GC-MS), respectively; 220 ºC injector temperature in split ratio of 1:30; fused silica capillary column (30 m × 0.25 mm); stationary phase Rtx®-5MS (0.25 µm film thickness); oven program with initial temperature of 40 °C for 3 min followed by graduated increments of 3 °C min^{-1} up to 180 °C, which was maintained for 10 min for a total analysis time of 59.67 min; and 240 ºC FID and 200 ºC MS detector temperatures (Souza et al., 2017;

Mendes et al., 2018). GC-MS analysis was performed in electronic impact equipment with 70 eV impact energy, 1,000 scan speed, 0.50 fragment sec^{-1} scanning interval, and fragment detection from 29 to 400 (m/z). GC-FID analysis was performed using an H_2 flame and atmospheric air at 300 °C. Flow speeds used for H_2 and air were 40 mL min^{-1} and 400 mL min^{-1}, respectively.

The identification of the EO components was performed by comparing the obtained mass spectra to those available in the spectral library database (Wiley 7, NIST 05, and NIST 05s) and the Kovats retention indices (KI). To calculate the KI, a sample of saturated alkanes C_7–C_{40} (Supelco, USA) and the adjusted retention time of each compound were employed as obtained by GC-FID. Subsequently, the values calculated for each compound were compared to those reported in the literature (Adams, 2007; NIST, 2011; El-Sayed, 2016). The percentage of each compound in the EO was calculated as the ratio between the integral area of the peaks and the total area of all sample constituents, as obtained from the GC-FID analysis. Compounds with a relative area > 1% were identified, and those > 5% were considered to be predominant.

Larvicidal activity

For larvicidal activity, three EOs (1, 2, and 3) from diploids, autotetraploids A, and autotetraploid B were selected based on lower, intermediate, and higher yields (%). The phytotoxicity and cytotoxicity bioassays were performed with two EOs (lower and higher yields) of the diploid autotetraploids A and B.

In order to verify the larvicidal activity of the EOs from diploids and autotetraploids *E. benthamii*, fourth-instar larvae of *A. aegypti* (PPCampos strain, Campos Goytacazes, RJ) were used. The larvae were obtained from a colony maintained at the Universidade Federal

de Viçosa. The eggs were incubated in trays with dechlorinated (tap) water, kept under controlled conditions (26 ± 2 °C, 12 h L:D photoperiod), and fed with turtle food (Reptolife®, Alcon,Camboriú, SC, Brazil) until the L4 stage (Who; 2005, modified by Mendes et al. 2017). EO solutions were prepared with 1% dimethyl sulfoxide (DMSO, v v^{-1}) at concentrations of 50, 75, 100, 125, 150, 175, and 200 ppm (Mendes et al., 2017). The larvae were collected with a Pasteur pipette and distributed into glass vials containing 30 mL of the EO solutions. Control solutions were prepared with 1% DMSO, as a negative control, and deltamethrin 0.07 ppm (Decis 25 EC, 25 g L^{-1}, emulsifiable concentrate, Bayer S.A., Germany), as a positive control. The assay was performed with five repetitions for each treatment with 25 larvae each (125 larvae per treatment), without solution replacement; after 24 h, *A. aegypti* mortality was assessed. The larvae were considered dead when no response to stimuli was found (direct contact with a Pasteur pipette). Relationships between different EOs and their concentrations were evaluated with a regression analysis (9 oils × 7 concentrations). A generalized linear model was used, with a Poisson distribution of residual effect and logarithmic ligation function. The following factors EOs, concentration, and its interaction were evaluated with deviance analysis at 5% probability of type 1 error. Confidence intervals of 95% of probability were built for each concentration of EO with the Effects package (Fox et al. 2015). All analyses were performed with R software (R Core Team 2019).

Phytotoxicity bioassay

A phytotoxicity bioassay was performed using EO concentrations tested at 187.5, 375, 750, 1,500, and 3,000 ppm, according to Aragão et al. (2015) and de Assis Alves et al. (2018). The statistical design was completely randomized with 5 replications for each treatment. Each experimental unit consisted of a Petri dish (10 cm × 2 cm) containing 25

seeds of *L. sativa* 'Itapuã Super' (Isla S.A., Brazil), which were placed on a piece of filter paper. EOs were dissolved in dichloromethane and applied using a sterile Pasteur pipette. The evaluated variables after exposure to EOs were as follows: (a) germination rate (GR, %) and root growth (RG, mm) after 48 h of exposure; (b) germination speed index (GSI), calculated every 8 h during 48 h, using the formula (N1 · 1) + (N2 - N1) · 1/2 + (N3 - N2) · 1/3 +... (Ny - (Ny - 1)) · 1/y (where Ny: number of germinated seeds in a given period; Y: total number of evaluations); and (c) shoot growth (SG, mm), determined after 120 h of exposure. The phytotoxicity bioassay was analyzed through a multiple linear regression with dummy variables, comparing each variable with the following factors: negative controls (water and dichloromethane), positive control (glyphosate), and EOs (diploid and autotetraploids A and B) as qualitative independent variables, and concentrations of EOs as quantitative independent variables. ANOVA for regression was performed at 5% probability and 95% confidence intervals were built for each concentration of EO with Effects package (Fox et al., 2015). The data were analyzed using R software (R Core Team, 2020).

Cytogenotoxicity bioassays

After 48 h in the phytotoxicity bioassay, 10 roots of *L. sativa* with the same, intermediate, and highest RG compared with controls, were fixed three times in fresh cold methanol:acid acetic acid (3:1), and stored overnight at -20 °C. The roots were fixed again and stored at -20 °C overnight in 70% ethanol. The influence of EOs on cell proliferation was verified with flow cytometry, comparing the number of G_2/M cells in root meristems of each treatment in relation to the positive and negative controls (water, dichloromethane, and glyphosate). Flow cytometry analysis was conducted with four repetitions; roots of five plants of each treatment were considered an experimental unit. Nuclei were isolated by macerating roots with a pestle five times in 700 µL Otto-I lysis buffer (Silva et al., 2010).

After 3 min, the suspensions were filtered through a 20-µm nylon mesh (Partec®, Germany) and centrifuged at 1,100 rpm for 5 min to separate the supernatant to be discarded. The pellets were eluted in 100 µL Otto-I lysis buffer for 10 min, and the nuclei suspensions were stained in 300 µL Otto-II solution (Otto, 1990) supplemented with 75 µM propidium iodide and immediately filtered through a 20-µm nylon mesh (Partec®). The homogenates were incubated in the dark for 20 min at 25 ± 1°C. The nuclei suspensions were analyzed in a BD Accuri C6 (Accuri cytometers, Belgium) equipped with a laser source to detect emissions at FL3 (> 670 nm). The histograms were analyzed using the BD CSampler™ software.

Additionally, clastogenic and aneugenic effects were evaluated from slides prepared from the same treatments used for cytometric analysis. Five previously fixed roots of *L. sativa* per treatment were used; each root was washed in dH_2O for 10 min, and subjected to hydrolysis in 5 N hydrochloric acid at 25 °C for 18 min. Each root meristem was placed on a glass slide with one drop of 2% orcein acetic solution, covered with a coverslip. Then, pressure was applied on the coverslip to macerate the root meristem (Aragão et al., 2015). The following variables were analyzed: (a) % of chromosomal alterations (AC), (c-metaphasis + anaphase bridges + chromosome fragments) / total number observed cells (10.000 cells); and (b) micronuclei occurrence.

3.4 Results

EO extraction, profiling, and germplasm characterization

Considering the stratification of leaves along the longitudinal axis, the EO yield (%) and chemical composition were the same. However, the yield and chemical composition of the EOs differed among the diploid and autotetraploid germplasm. Autotetraploid A showed higher yields (1.71–7.21%; w w^{-1}) compared to the diploids (1.08–2.3%; w w^{-1}) and

autotetraploid B (0.65–1.98% w w^{-1}). Despite the ploidy level difference, the EO yield was the same between the diploids and the autotetraploid B (Table 1).

Gas chromatography showed that the diploids EOs presented α-pinene (60.43%), viridiflorol (19.93%), and aromadendrene (19.64%); both autotetraploids A and B EOs presented 1,8-cineole (54.76%) and α-pinene (31.98%) as the major compounds. The compounds aromadendrene and viridiflorol present in the diploid EOs were not identified in autotetraploid EOs. In addition, autotetraploid EOs presented different compounds: autotetraploids A, α-terpineol (13.21%) and autotetraploids B, α-terpinyl-acetate (8.46%) and limonene (4.75%). A single sample from autotetraploid B also exhibited trans-β-ocimene (0.56%). The quantitative variation (%) among aromadendrene, viridiflorol, and 1,8-cineole had a positive correlation with yield (%) for each germplasm (0.62, 0.35, and 0.23, respectively) (Table 2).

Table 1: Yield (%) and chemical composition (%) of the *E. benthamii* EOs. Diploid and Autototetraploids A and B germplasm were vegetative propagated. Identification 1, 2 and 3 refers to yield (lower, intermediate and higher, respectively).

Germplasm	Ploidy	Yield	aromadendrene	1,8-cineole	limonene	viridiflorol	α-pinene	α-terpineol	α-terpinyl acetate
diploid 1	2x	1.16	16.92	-	-	19.78	63.3	-	-
diploid 2	2x	1.75	22.1	-	-	18.74	59.16	-	-
diploid 3	2x	2.30	22.61	-	-	21.83	55.56	-	-
autotetraploid A1	4x	1.79	-	58.72	-	-	29.21	12.07	-
autotetraploid A2	4x	4.31	-	54.32	-	-	33.17	12.51	-
autotetraploid A3	4x	7.21	-	55.93	-	-	31.14	12.93	-
autotetraploid B1	4x	0.64	-	52.10	5.14	-	34.13	-	8.63
autotetraploid B2	4x	1.73	-	51.19	5.47	-	35.49	-	7.85
autotetraploid B3	4x	1.98	-	49.78	5.10	-	31.45	-	8.62

Table 2: Chemical identification of the compounds present in diploid and autotetraploid *E. benthamii* germplasm.

Germplasm	Ploidy	Compound	Mean (%)	Yield (%)	r (compound/yield)	n	t calc	t tab	Significance at 5%
Diploid/ A/ B	2x/4x/4x	α-pinene	-	-	-0.2550407	27	-1.319	0.9605	ns
A/B	4x/4x	1,8-cineole	-	-	0.2345942	18	0.9653	0.9607	*
Diploid	2x	aromadendrene	19.63 ± 2.38	1.56	0.6242518	9	2.1141	0.9615	*
		viridiflorol	19.94 ± 1.50	1.56	0.3518146	9	0.9944	0.9615	*
A	4x	α-terpineol	12.7 ± 0.70	3.05	0.1519108	9	0.4066	0.9615	ns
B	4x	limonene	4.75 ± 1.79	1.23	0.1018077	8	0.2507	0.9617	ns
		trans-β-ocimene	0.56 ± 1.68	1.23	-	1	-	-	-
		α-terpinyl acetate	8.42 ± 0.57	1.23	-0.1324388	9	-0.354	0.9615	ns

Larvicidal bioassay

Diploid EOs ranging from 50 to 200 ppm showed larvicidal activity. EOs from diploid 2 and 3 (intermediate and higher yields) promoted 50% of larval death at 100 ppm and 80% at 150 ppm. EOs from diploid 2 and 3 showed 5.18% and 5.69% more aromadendrene, and 4.14% and 7.74% less α-pinene than diploid 1 EOs. Diploid 1 (lower yield) and autotetraploids A and B' EOs promoted 50% of deaths only at 200 ppm (Figure 1). The positive control solution with Deltamethrin caused 100% mortality; thus, treatments with 25 dead larvae had the same effect as the positive control. The negative control (DMSO) did not promote any deaths, indicating that DMSO, used as a solvent, had no relation to the larvicidal effect of EOs.

Phytotoxicity bioassay

Most EOs from *E. benthamii* diploid and autotetraploids did not significantly reduce the GR of *L. sativa* seeds. Despite autotetraploid A1 at 750 and 1500 ppm (means = 97.65% and 96.32%, respectively), autotetraploid B3 at 1,500 ppm (mean = 97.83%), and diploid 1 at 3000 ppm (mean = 96.82%) were statistically different from the positive and negative controls (mean = 100%), GR caused by these EOs was still considered high for this bioassay (Figure 2). Autotetraploids A1, B3, and diploid 1 (from 375 ppm to 3,000 ppm) also promoted a reduction in GSI (Figure 3) when compared to the water control (GSI = 10.66). Seeds treated with autotetraploid B3 and diploid 1 (3,000 ppm) reached GSI = 7.6 and 7.94, respectively.

EOs from autotetraploid germplasm A1, A3, and B1 promoted RG at all concentrations. Autotetraploid B1 promoted higher RG (18.18 mm) than the water control (RG = 10.97). Seeds treated with diploid 3 and autotetraploid B3 EOs did not differ from the controls, while diploid 1 caused a reduction in RG from 187.5 ppm (15.45 mm) and to started

inhibition at 3,000 ppm (8.7 mm) (Figure 4). However, EOs had an evident effect on post-emergence of *L. sativa* plantlets, causing a reduction of up to 6.2 mm in the SG (Figure 5). Autotetraploids B1 and B3 and diploid 3 EOs had the same effect as glyphosate herbicide (SG = 0.25 mm). Autotetraploid A1 and A3 EOs also reduced SG as well as diploid 1, which it was gradually (Figure 5).

Cytogenotoxicity bioassay

Based on the mean number of G_2/M nuclei (4C and 8C), the root apical meristem cells showed the same proliferation rate among all treatments. Thus, the EOs of the autotetraploid strains A3 and B1 and diploid 1 from 750 to 3,000 ppm were not statistically different from the positive or negative controls (Figure 6). In cytotoxicity and genotoxicity assays, no chromosome alterations, nuclear disorders, or micronuclei formation were observed. The cells presented well-preserved nuclei and normal mitotic phases even at EOs highest concentration (3,000 ppm).

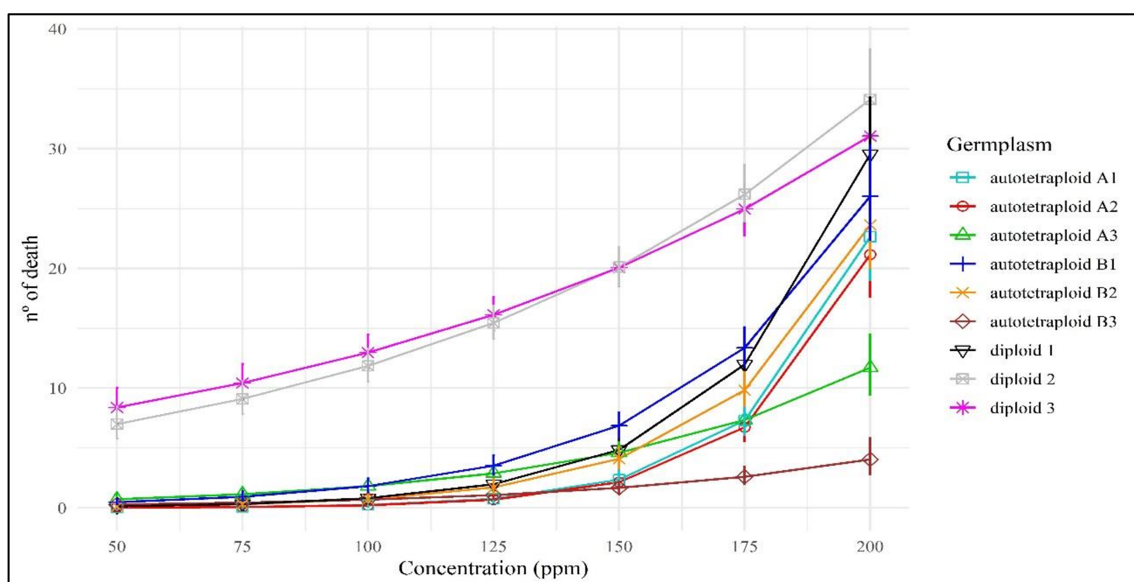


Figure 1: EOs from *E. benthamii* diploid and autotetraploid germplasm tested in Larvicidal bioassay. Number of dead *A. aegypti* larvae after 24 h of exposition at EOs different concentrations (positive control: Deltamethrin; negative control: DMSO).Germplasm: diploid 1, 2 and 3 (lower, intermediate and higher yield,

respectively); autotetraploid A1, A2 and A3 (lower, intermediate and higher yield, respectively); autotetraploid B1, B2 and B3 (lower, intermediate and higher yield, respectively). The bars are 95% confidence intervals. Overlapped confidence intervals indicates that statistical difference was not detected.

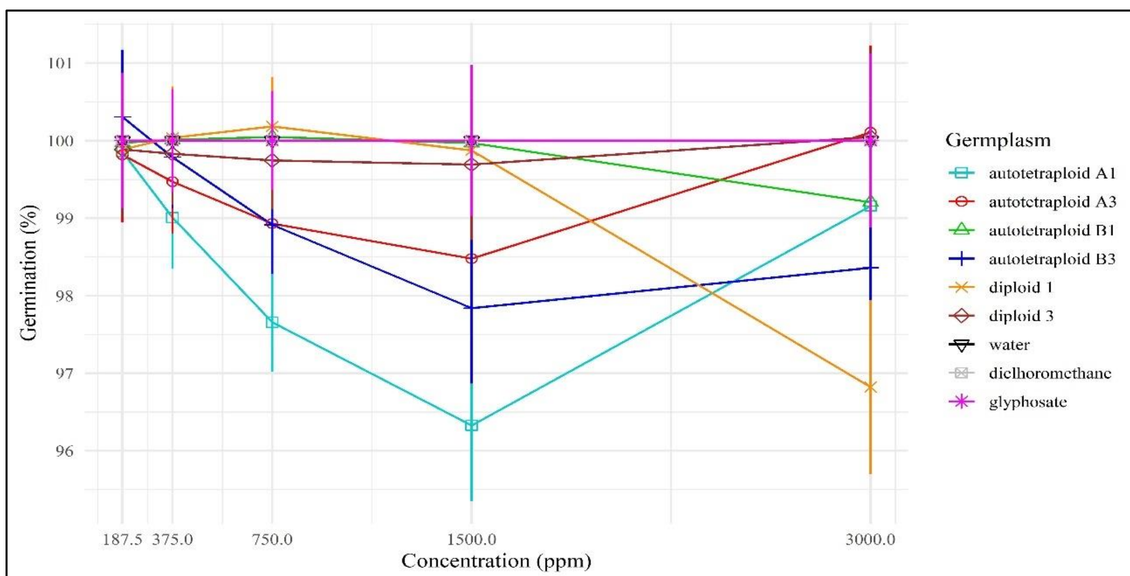


Figure 2: EOs from *E. benthamii* diploid and autotetraploid germplasm testing GR (%) of *L. sativa* seeds after 48 h of exposition. Germplasm: diploid 1, 2 and 3 (lower, intermediate and higher yield, respectively); autotetraploid A1, A2 and A3 (lower, intermediate and higher yield, respectively); autotetraploid B1, B2 and B3 (lower, intermediate and higher yield, respectively). The bars are 95% confidence intervals. Overlapped confidence intervals indicates that statistical difference was not detected.

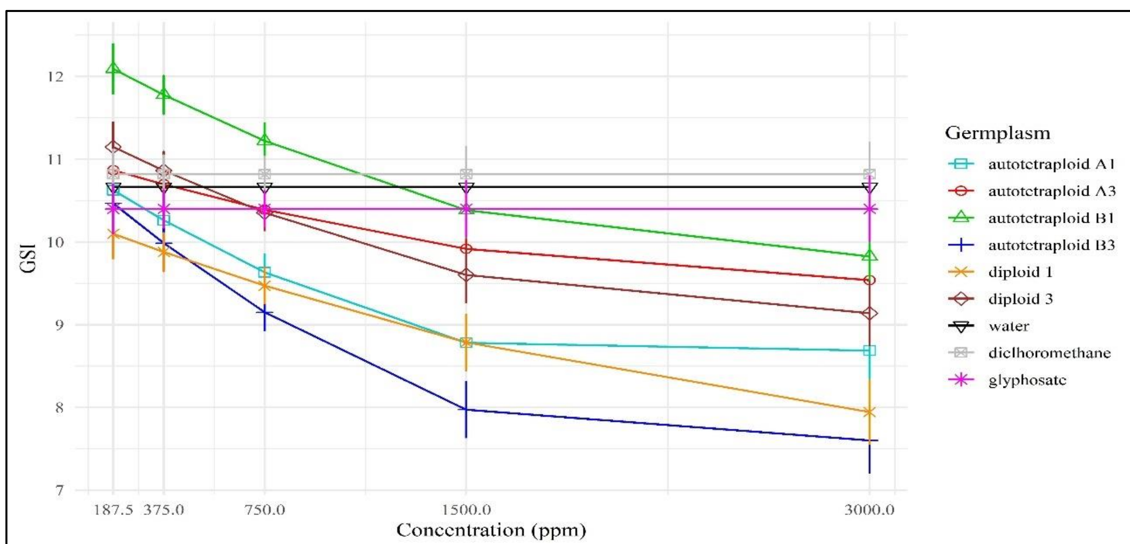


Figure 3: EOs from *E. benthamii* diploid and autotetraploid germplasm testing GSI of *L. sativa* seeds after 48 h of exposition. Germplasm: diploid 1, 2 and 3 (lower, intermediate and higher yield, respectively); autotetraploid A1, A2 and A3 (lower, intermediate and higher yield, respectively); autotetraploid B1, B2 and B3 (lower, intermediate and higher yield, respectively). The bars are 95% confidence intervals. Overlapped confidence intervals indicates that statistical difference was not detected.

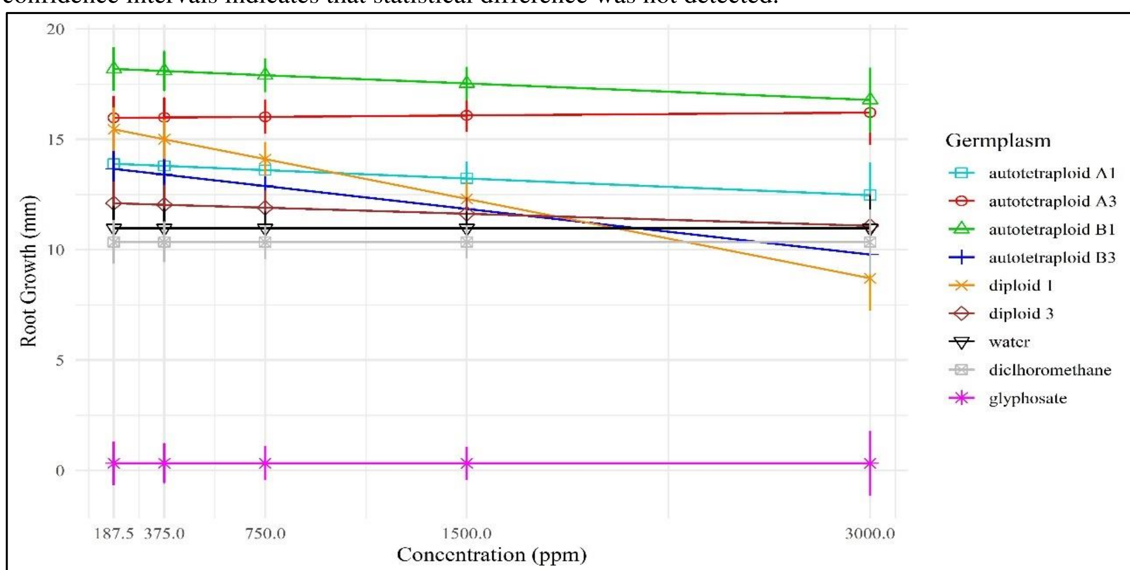


Figure 4: EOs from *E. benthamii* diploid and autotetraploid germplasm testing RG (mm) of *L. sativa* seeds after 48 h of exposition. Germplasm: diploid 1, 2 and 3 (lower, intermediate and higher yield, respectively); autotetraploid A1, A2 and A3 (lower, intermediate and higher yield, respectively); autotetraploid B1, B2 and B3 (lower, intermediate and higher yield, respectively).

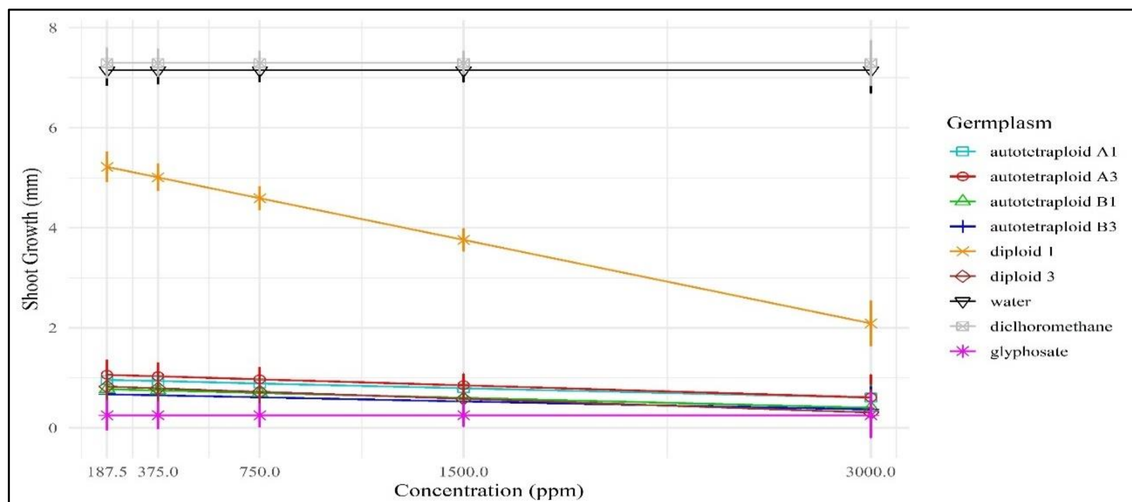


Figure 5: EOs from *E. benthamii* diploid and autotetraploid germplasm testing SG (mm) of *L. sativa* seeds after 48 h of exposition. Germplasm: diploid 1, 2 and 3 (lower, intermediate and higher yield, respectively); autotetraploid A1, A2 and A3 (lower, intermediate and higher yield, respectively); autotetraploid B1, B2 and B3 (lower, intermediate and higher yield, respectively).

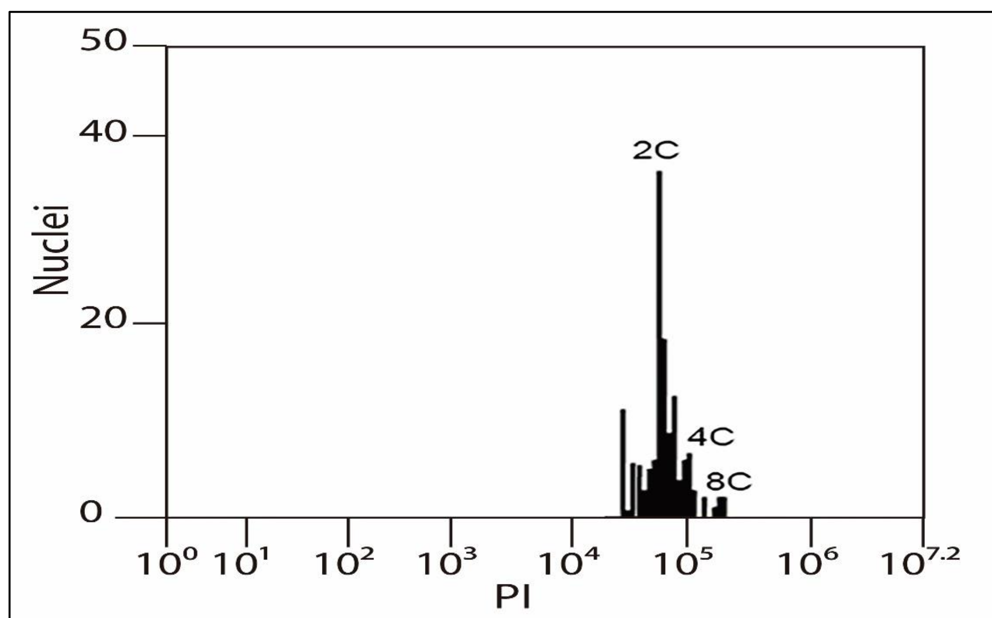


Figure 6: Representative flow cytometry histogram of the root apical meristem cells after 48 h of EO exposition. Peak 2C represents diploid nuclei of L. sativa (2n = 2x = 18 chromosomes; Koopman et al., 1993) in G0/G1 of the cell cycle. Peak 4C represents diploid nuclei in G2/M and autotetraploid nuclei in G0/G1. Peak 8C represents autotetraploid nuclei in G2/M.

3.5 Discussion

Ploidy level and chemical composition of EOs

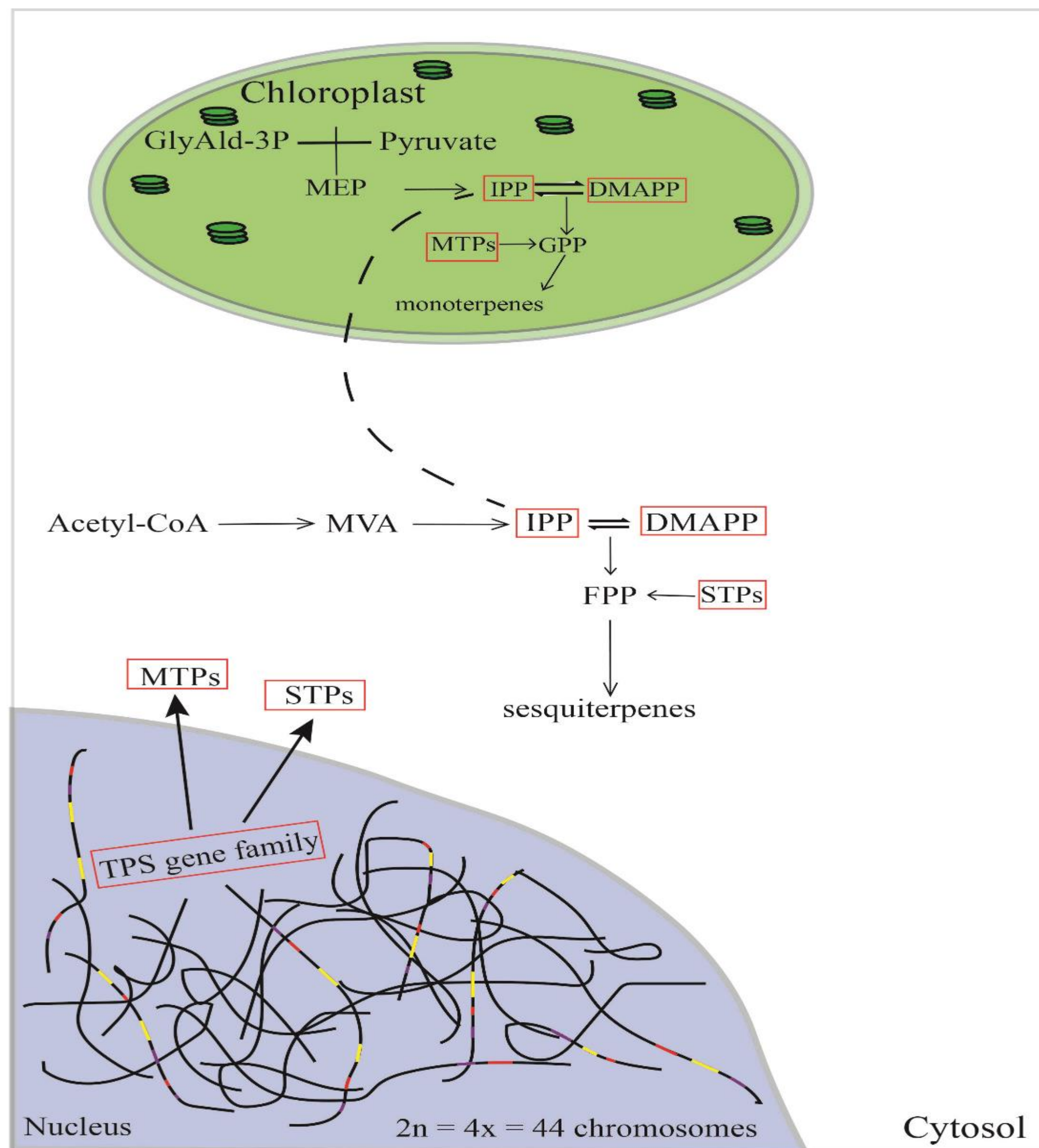
Autotetraploidy promoted chemical diversity in *E. benthamii* EOs, as proved by the occurrence of monoterpenes such as 1,8-cineol, limonene, α-terpineol, and α-terpinyl acetate, which not found previously in *E. benthamii* diploid germplasm. Qualitative differences in EOs were also described in autotetraploid *Trachyspermum ammi* L., in which the EOs extracted from diploid accessions did not present α-terpineol (Noori et al., 2017). In *C. limon* EOs, autotetraploids produced β-bisabolene, but this compound was absent in diploid EOs (Bhuvaneswari et al., 2020). The changes in EO composition are possibly associated with new gene expression profiles and enzyme activities, which modulate the biosynthesis of secondary metabolites (Iannicelli et al., 2020). In this study, we also reported a quantitative change in the α-pinene content of autotetraploids A and B EOs compared with their diploid relatives. Bhuvaneswari et al. (2020) also identified these changes in *C. limon* autotetraploids. The authors attributed the changes to an upregulation of the limonene synthase gene, which led to an increase of 22.23% in limonene content.

The ratio between monoterpenes and sesquiterpenes was also altered in EOs of *E. benthamii* autotetraploids, as they did not contain aromadendrene and viridiflorol sesquiterpenes. The alteration in terpenes derived from secondary metabolism has been described as an euploidy effect, indicating that the increase in ploidy level can modify pathways differently (Iannicelli et al., 2020). Considering that *E. benthamii* is predominantly allogamous, the differences in EOs compositions among the autotetraploid germplasm indicate that the genotype also contributed to the chemical diversity. Furthermore, there are 113 genes related to terpene synthases in the *E. grandis* genome (Chen et al., 2011), and the autotetraploidy increases the number of gene copies per locus.

Hence, autotetraploidy may alter the chemical composition and ratio of mono and sesquiterpenes differently, according to the pathway explored (Figure 7). Potentially, CSD would double all the TPS gene family in the nucleus, and consequently, a new gene expression pattern would be set. As antimitotic agents used for CSD only act as spindle inhibitors (Dhooghe et al., 2011), the dynamics of gene expression and enzyme activity are related to the doubled nuclear genome. Moreover, monoterpenes predominantly originate from the MEP pathway in chloroplasts, whereas sesquiterpenes, in the cytosol and peroxisomes (Aharoni et al., 2005). So far, the CSD effect in *E. benthamii* supported the MEP pathway, possibly producing more monoterpene synthases that were imported to the chloroplast (Fig. 7). Additionally, geranyl diphosphate synthases compete for the precursor IPP and DMAPP to produce different monoterpenes in chloroplasts (Bouvier et al., 2000; Külheim et al., 2011). Thus, the autotetraploid condition could enhance the precursor availability of IPP and its isomer, DMAPP, as well as geranyl diphosphate synthases in

chloroplasts. This would possibly explain the dominance of monoterpene compounds in EOs (Fig. 7).

Figure 7: The terpene pathway in E. benthamii autotetraploids. Solid arrows indicated multiple enzymatic and



transport steps. Red boxes indicate the role of chromosome set doubling in terpene biosynthesis. Long dashes represent the possible crostalk between cytosol and chloroplast of the precursor IPP. Abberviations: TPS, terpene synthase gene family; MTPs, monoterpene synthases; STPs, sesquiterpene synthases; MVA, mevalonic acid; IPP, isopentenyl diphosphate; DMAPP, dimethilallyl diphosphate; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; MEP, methylerythritol 4-phosphate; GlyAld-3P, glyceraldehyde 3-phosphate.

Therefore, it is possible that autotetraploids have an overdominance effect over diploids, leading to diverse phenotypic changes (Ramsey and Schemske, 2002). The

presence of 1,8-cineole, limonene, and α-terpineol has been reported for diploid *E. benthamii* in different Brazilian locations (Mossi et al., 2011; Döll-Boscardin et al., 2012); in the present study, these compounds did not occur in diploid germplasm, and the trees were randomly planted in the same field. It is possible that the diploid germplasm did not produce 1,8-cineole, limonene, and α-terpineol because of the quantitative effect of genes that regulate essential oil concentrations. In fact, many quantitative trait *loci* are associated with terpene production *of E. nitens* and *E. globulus* (Henery et al., 2007; Külheim et al., 2011), some with larger and others with smaller regulatory effects (Kainer et al., 2015), which could explain the production of terpenes in different proportions when the genome is doubled.

Larvicidal activity of diploid and autotetraploid EOs

Diploid EOs that showed high toxicity had a higher percentage of aromadendrene and viridiflorol, indicating that these sesquiterpenes may have a crucial role in larvicidal activity against *A. aegypti*. Lucia et al. (2008) studied 12 *Eucalyptus* species and found a correlation between EOs yield and 1,8-cineole, the higher the percentage of 1,8-cineole the lower the mortality rates of *A. aegypti* larvae. These results could explain the lower toxicity of *E. benthamii* autotetraploid EOs, as they have 1,8-cineole as major compounds and only monoterpenes in its composition. Organophosphate-based insecticides such as temephos are efficient at eradicating *A. aegypti* larvae in concentrations lower than 1 ppm, but these insecticides frequently affect non-target organisms and also lead to larval insecticide-resistance (Braga et al., 2004). The chemical diversity of EOs provides a natural barrier against insect resistance due to its chemical complexity (Barbosa et al., 2016), and the diploid EOs of *E. benthamii* can be used as natural biomolecules.

In addition to the lower toxicity, chemical variability presented by autotetraploid EOs may show advantages over their diploid counterparts. Hantao et al. (2013) identified 1,8-

cineole and α-terpynil acetate as biomarkers for myrtle rust resistance in hybrids of *E. grandis* × *E. urophylla.* Moreover, EOs are involved in plant defense against herbivory, pathogens, and environmental interactions (Chen et al., 2011; Te Beest et al., 2012). So far, autotetraploid trees could be used for *Eucalyptus* improvement aimed at plant defense against myrtle rust disease.

EOs post-emergence activity in L. sativa

Considering the phytotoxicity bioassay, there was no influence of EO exposure in diploid autotetraploids A and B regarding percentage of germination and GSI. In RG, autotetraploids A and B showed an increase in growth (> 5 mm) compared with water controls. Batish et al. (2006), using 130 ppm of *C. citriodora* EO, reported a root elongation effect in *Amaranthus viridis* and *Echinochloa crus-galli*, revealing that EOs in *Corymbia* and *Eucalyptus* can cause this effect in different plant roots.

In general, EOs from diploid and autotetraploid *E. benthamii* had an inhibitory effect on SG. However, autotetraploids B1 and B3, and diploid 3 had the same effect as the positive control, glyphosate. The major compounds, α-pinene, found in all germplasm, and 1,8-cineole (autotetraploid germplasm) are also known to inhibit SG in *L. sativa* plantlets under *E. grandis* EOs (Aragão et al., 2015). As EOs are complex substances, their phytotoxic activity is possibly related to synergism between monoterpenes (Arminante et al., 2006). Moreover, the non-effect on RG and the remarkable effect on SG indicate that *E. benthamii* EOs act as a post-emergence weed controller, reducing plantlet development. Batish et al. (2007) studied the effect of *C. citriodora* EOs, and the authors demonstrated the inhibition of SG, caused by the reduction in chlorophyll content, and the severe inhibition of photosynthesis and respiration. In addition, cell electrolyte activity under *C. citriodora* EOs

was enhanced, which possibly altered membrane permeability, leading to oxidative stress (Daizy Rani Batish et al., 2007).

Cytogenotoxicity: DNA damage and mitotic agents

Phytotoxicity bioassays generally show the allelopathic potential of EOs over seed germination and EOs influence on plantlet growth and development (Vasconcelos et al., 2019), Moreover, such bioassays can indicate changes in DNA (Prates et al., 2000). In the present study, we evaluated cell proliferation in the roots of *L. sativa* plants treated with control and EO solutions; no significant differences were found in G1 or G2/M phases. These results indicate that the tested EOs are not mitotic promoters that would favor cell division. Moreover, the diploid and autotetraploid EOs of *E. benthamii* did not promote any chromosome alteration or micronuclei formation, which would cause genomic damage. Clastogenic and aneugenic agents cause DNA breaks and dysfunctional chromosome segregation (Fernandes et al., 2007), reducing the mitotic index and, consequently, reducing RG (Andrade et al., 2010). As those agents were not identified in *L. sativa* roots, *E. benthamii* EOs only showed phytotoxic activity at the initial stages of SG development.

3.6 Conclusions

CSD has been applied in forest species to attend breeding programs. Because trees have long cycle times, it is urgent to describe the effects of CSD in early development. Autotetraploids of *E. benthamii* produced 1,8-cineole, limonene, α-terpynil acetate, and α-terpineol, not previously identified in diploid EOs. These chemical changes were related to the increase in ploidy levels and its many effects in the “omics” environment. Hence, the genotype differences also contributed to the chemical diversity (autotetraploid A produced

α-terpineol, while autotetraploid B, limonene and α-terpinyl acetate). Despite the diversification in EO compounds, the autotetraploid condition did not increase the EO yield (autotetraploid B had the same yield of diploid germplasm).

Exploring the context of chemical diversity in bioassays, diploid germplasm were 2-fold more lethal against *A. aegypti* larvae than autotetraploids. This could be explained by the sesquiterpenes constitution of the diploid EOs, while autotetraploids only produced monoterpenes. In phytotoxicity bioassays, the major effect of EOs was on SG, acting as a post-emergence controller in *L. sativa* and compared to the glyphosate herbicide. Thus, the EOs of diploid and autotetraploid germplasm of *E. benthamii* have an impact only in the aerial parts of plants, because there was neither genotoxic nor antimitotic properties on cell proliferation in root apical meristems.

Therefore, the increase in ploidy level leads to chemical diversity in *Eucalyptus* secondary metabolites, altering the ratio of mono/sesquiterpenes in EOs. Despite the low toxicity of autotetraploid EOs against *A. aegypti* larvae, 1,8-cineole and α-terpynil acetate, present in autotetraploids EOs, were linked as biomarkers to myrtle rust disease. Furthermore, phenotypic selection through the autotetraploid germplasm is required to achieve a greater quantity of EO yield and specific major compounds, considering the scope of the breeding program.

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4. RESEARCH PAPER 2: Inducing DNA demethylation in *Eucalyptus urophylla* × *Eucalyptus grandis*: new epigenetic germplasm?

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4.1 Abstract

DNA methylation is one of many epigenetic processes known for plant development success. Such changes in DNA methylation are able to regulate from gene expression to phenotype variation, being stable and therefore transmitted cell to cell and to the offspring. As far as we understand how epigenetic changes occurs, and characterize its effects, it has been possible manipulate these marks across genome by demethylating it with chemicals such as 5-Azacytidine (5-AzaC). In the present work, we germinated seeds of *Eucalyptus urophylla* × *Eucalyptus grandis* under 5-AzaC concentrations (0, 0.5, 1.0, 5.0, 10, 30 and 50 µM), and induced root formation under 5-AzaC without growth regulators. Another experiment we induced the same germinated plants to shoot formation using hypocotyls and cotyledons as explants. In the rooting experiment, plants treated with 0.5, 1.0, 5.0 and 10 µM 5-AzaC created more roots than controls, the best treatment was 1 µM resulting in a 65% of root increasing. Hypocotyls and cotyledons produced 99% of callus and 3 to 10% of shoots at the callus formation phase (in the dark). Hypocotyls callus in 5.0 µM and then regenerated in 5-AzaC free media generated more 6.5 shoots/explant, while cotyledons callus in 10 µM regenerated 9.5 shoots/explant. Since materials had high responsiveness, we formulated a new media to recover a maximum number of treated plantlets. Hypocotyls subsequently treated with 5.0 µM 5-AzaC yielded 227 plants and cotyledons with 1.0 µM yielded 183 plants. 5-AzaC brought about the 5-methyl cytosine levels (%) in all experiments, being evident the demethylation using 5.0 µM, as germinated hypocotyls presented 48% of methylated cytosines, 23.5% at callus stage and 15.3% in regenerated plants. The application of 5-AzaC as demethylating agent improve rooting and shooting. Therefore, its use in plant breeding and plant biotechnology may restore responsiveness of germplasm and even amplify the phenotype variability, which would be the later subject of these research.

4.2 Introduction

Plant growth and development, as the responses to biotic and abiotic stress, have a thin and coordinated control of which gene must be expressed, when and where (spatial and temporal) to ensure the proper response of plants to stimuli. For such regulations, DNA methylation has many functions in plant metabolism, *i.e.* silencing transposable elements, changes in gene expression and chromatin structure (heterochromatin patterns along chromosomes) (Ikeuchi et al., 2015; Saze et al., 2012). Cytosine DNA methylation (5mC) in particular, is an epigenetic modification process that occurs through addition (by covalent bond) of methyl radical at 5' of the pyrimidine ring of a cytosine, resulting in a 5-mehtyl cytosine (5-mC) (Us-Camas et al., 2014). These epigenetic changes are replicated by DNA methyltransferases during the DNA replication, thereby inherited by the offspring (cells, tissues, germinative cells and progenies) allowing the cells to "remember" the parental previous conditions, also called epigenetic memory (Birnbaum and Roudier, 2017).

Thus, the 5-mC levels are very distinct in many tissues and organs, as well as the epigenome when plants are subjugated to different environment conditions (Alonso et al., 2018). From a breeding perspective, epigenetic modifications (natural or induced) must be stable when inherited by the offspring (Gallusci et al., 2017). These epigenetic marks can be replicated during mitotic cell division and transmitted from cells to tissues (Lee and Seo, 2018). Thereby, the phenotype plasticity (ability of the same genotype demonstrate distinct phenotypes in different environments) may be attributed to DNA methylation changes. This may be a crucial output in plant breeding of vegetative propagated species, once the DNA methylation triggers many process and physiological responses in plant development and growth, amplifying the phenotype diversity (Latutrie et al., 2019).

The pivotal role of DNA methylation has been reported for *E. urophylla* × *E. grandis* clonal hybrids (namely as *E. urograndis*), in wet and dry locations. Pereira et al., (2020)

found 445 methylated sites significantly correlated with one growth trait (volume, height or diameter at breast height), and so far, different patterns of DNA methylation among the clones. In *Malus domestica* Borkh., seedlings, young grafts and old grafts had the same %5-mC levels, but remarkable differences in transcriptomics profiles. Differentially methylated sites analysis revealed that older materials have more methylation close to genes and transposable elements, evidencing a clear methylation pattern associated to transition of juvenile to mature tissues (Perrin et al., 2020). Other characteristics has also been explored such fruit ripening in tomato (Liu et al., 2015), increased seed protein and decreased oil content in canola (Long et al., 2011), as well as indirectly in productivity through photosynthetic genes such as RuBisCO (ribulose-1,5-biphophate carboxylase oxygenase) and PEPC (phosphoenolpyruvate carboxylase) (Duarte-Aké et al., 2019).

As DNA methylation have different roles in plant development, its manipulation has also been explored artificially through chemical compounds. The well-studied 5-Azacytidine (5-AzaC), is a cytosine analogue that can be incorporated into DNA strands each cycle of DNA replication. Once incorporated, they form a covalent complex with the DNA methyltransferases, depleting its activity and resulting in an hypomethylation condition that is propagated during replication (Griffin et al., 2016). Strategically, 5-AzaC can be used in tissue culture media in order to create epimutants (Munsamy et al., 2013), germinated plants with new 5-mC status (Massoumi et al., 2017) or even to improve and understand complex morphogenic process such somatic embryogenesis (Fraga et al., 2012; Osorio-Montalvo et al., 2020).

Further, choosing the right method for identification and quantification of DNA methylation depends what kind of question needs to be answer (Kurdyukov and Bullock, 2016). High Performance Liquid Chromatography in tandem with mass spectrometry (HPLC-MS), provides a precise status of the global DNA methylation, or whole genome

methylation profiling, and is used for prospective studies, stress and induced DNA demethylation with chemical such as 5-AzaC (Fraga et al., 2012; Osorio-Montalvo et al., 2020). For more precise epigenetic modifications, identifying specific methylated regions or demethylating targeted genes, bisulfite sequencing is recommended for such approaches (Kurdyukov and Bullock, 2016).

Aiming to explore possible traits by demethylating DNA, we used 5-AzaC for generate a *Eucalyptus urograndis* germplasm with divergent DNA methylomes, that share a similar genomic background. These hypomethylated germplasm were generated through organogenesis.

4.3 Materials and Methods

Plant material and explant source for demethylation experiments

Open-pollinated seeds of *E. urograndis* from Klabin Experimental Research Station (Klabin S. A., Parana State, Brazil) were surfaced disinfected with 70% ethanol for 1 min, 0.2% of NaClO supplemented with 2 drops of Tween 20 for 20 min, and washed 3 times in deionized sterile water for 5 min each. The seeds were germinated in germination media WPM basal salts media (Sigma®) supplemented with 10 m L^{-1} MS vitamins (stock solution: 0.2 g L^{-1} glycine (Sigma®), 0.05 g L^{-1} nicotinic acid (Sigma®), 0.05 g L^{-1} pyridoxine (Sigma®) and 0.01 g L^{-1} thiamine(Sigma®)), 0.1 g L^{-1} Myo-inositol (Sigma®),0.05 g L^{-1} L-cystein (Sigma®), 30 g L^{-1} Sucrose (Sigma®), 2.8 g L^{-1} Phytagel (Sigma®), pH 5.8 and previously autoclaved for 20 min at 121°C and 121 kPa. 5-AzaC at 0.5, 1.0, 5.0, 10, 30 and 50 µM filter-sterilized was added to the media (at 45 °C) before pouring to the test tubes.

Germination and rooting under 5-AzaC

Once germinated, 15-days old cotyledons, hypocotyls and roots were collected in bulk (each containing 10 explants/sample, total of 10 samples) and the global %5mC status were checked. These plantlets were used as source of explants to organogenesis experiments and the remaining were cultivated again for 30 days in the fresh germination media. These plants were divided in two groups: a) inoculated in media supplemented with the same 5-AzaC concentrations, and b) inoculated 5-AzaC free. Both groups had their roots excised before inoculation. After 30 days, leaves were collected for %5mC analysis, and the ability of adventitious rooting without growth regulators were evaluated by counting the number of roots/plant.

Callus induction, shooting and plantlet recovery

After germination, hypocotyls and cotyledons of 15-days old plantlets were excised and inoculated in petri dishes with 20 mL of Callus Induction Media (CIM).The media was suggested by França Bettencourt et al. (2020), with the following modifications: 2.5 µM BAP + 0.5 µM NAA, 0.1 g L^{-1} Myo-inositol (Sigma®), 0.05 g L^{-1} L-cystein (Sigma®), MS vitamins, 2.8 g L^{-1} Phytagel (Sigma®) and 5-AzaC at 0.5, 1.0, 5.0, 10, 30 and 50 µM. The cultures were kept in the dark, at 25 ± 1°C for 30 days. A total of 20 replicates for hypocotyls and 10 for cotyledons explants were set (each replication with 10 explants/petri dish). Following, the number of callus, oxidation (browning) and shoot number were evaluated. Ten callus/treatment were collected in bulk for epigenetic analysis.

After callus formation, the masses were transferred to pretri dishes containing 20 mL of Shoot Induction Media (SIM) according to França Bettencourt et al. (2020), with the following modifications: 5 µM BAP + 1 µM NAA, 0.1 g L^{-1} Myo-inositol (Sigma®), 0.1 g L^{-1} L-cystein (Sigma®), 0.1g L^{-1} L-Ascorbic acid, 0.05 g L^{-1} Polyvinylpyrrolidone, 10 m L^{-1}

MS vitamins, 2.8 g L^{-1} Phytagel (Sigma®) and 5-AzaC at 0.5, 1.0, 5.0, 10, 30 and 50 µM. Ten replicates of hypocotyls from the CIM step were subcultured in 5-AzaC free. The callus were cultivated for 30 days in a growth room at 25 ± 1°C. Considering the degradation of 5-AzaC, the explants were subcultured into a fresh medium each 15 days, for 30 days. After 30 days the number of responsive callus, shoots and number of shoots/explant were evaluated.

Shoots from SIM were excised from the responsive callus and transferred to a Plantlet Recovery Media (PRM) for a brief elongation: WPM basal salts media (Sigma®) supplemented with 0.5 µM BAP + 0.01 µM NAA, 10 m L^{-1} MS vitamins, 0.1 g L^{-1} Myo-inositol (Sigma®), 0.05 g L^{-1} L-cystein (Sigma®), 0.1g L^{-1} L-Ascorbic acid, 0.05 g L^{-1} Polyvinylpyrrolidone, 30 g L^{-1} Sucrose (Sigma®), and 2.8 g L^{-1} Phytagel (Sigma®) at pH 5.8. After 30 days in PRM media, three plantlets/treatment 1 cm long were collected for % 5-mC measurements.

Global DNA methylation levels

To access the hypomethylation condition of the explants, % 5-mC levels were measured through High Proficiency Liquid Chromatography (HPLC). Hypocotyls and cotyledons of plantlets used as explant donors, the callus masses grown in CIM, shoots recovered in PRM and leaves of the germinated plants (same group used as explant source) were collected and stored at – 80 °C until genomic DNA extraction. The experimental design of organogenesis under 5-AzaC was stated in Fig. 1. Samples were macerated in MagNa Lyser (Roche®) and genomic DNA was extracted and purified according to Doyle and Doyle (1990), and eluted in H_2O Type I. The DNA concentration was estimated using a NanoDrop (ThermoScientific® 2000c).

A total of 15 µg of DNA of each sample was hydrolyzed in 70 % perchloric acid ($HClO_4$) and heated 100 °C in dryed bath for 60 minutes. The pH was adjusted between 3 and 5 with 1.0 mol L^{-1} potassium hydroxide (KOH), following a centrifugation at 1000 rpm for 5 minutes (Chen et al., 2013). The supernatant was transferred to a new microtube and the hydrolyzed solvent was evaporated in a sample concentrator (Speed Vac, Thermo Scientific®). The samples were stored at 5 °C until use.

The hydrolyzed DNA was eluted in 50 µL H_2O Type I and injected in a HPLC system (model Shimadzu® LC-20AT), equipped with a photodiode array detector (Shimadzu SPD-M20A) and C18 stainless steel column (Zorbax Eclipse XDB-C18, 250 mm x 4.6 mm I.D., 5-µm particle size; Agilent®). The aqueous solution of mobile phase contained 50 mmol L^{-1} ammonium phosphate dibasic $(NH_4)_2HPO_4$ was previously vacuum filtered in a 0.45 µm nylon filter on ultrasound for 10 minutes. The flow was 0.5 mL/minute and the monitored wavelength was 270 nm (Chen et al., 2013).

The determination of genomic content of 5-methylcytosine was performed by external standard of cytosine and 5-methylcytosine (Sigma®) and determined by the equation: $\%(5\text{-mC}) = (5\text{-mC}/C + 5\text{-mC}) \times 100$, where 5-mC is the concentration of 5-methylcytosine (µmol L^{-1}), and C is the concentration of cytosine (µmol L^{-1}).

Statistical analysis

ANOVA was performed in the rooting and shooting experiments, considering the number of roots formed in both 5-AzaC and 5-AzaC free media; and the explant responsiveness (hypocotyls and cotyledons) regarding the number of shoots. The averages were compared at 5% of probability by the Tukey's test.

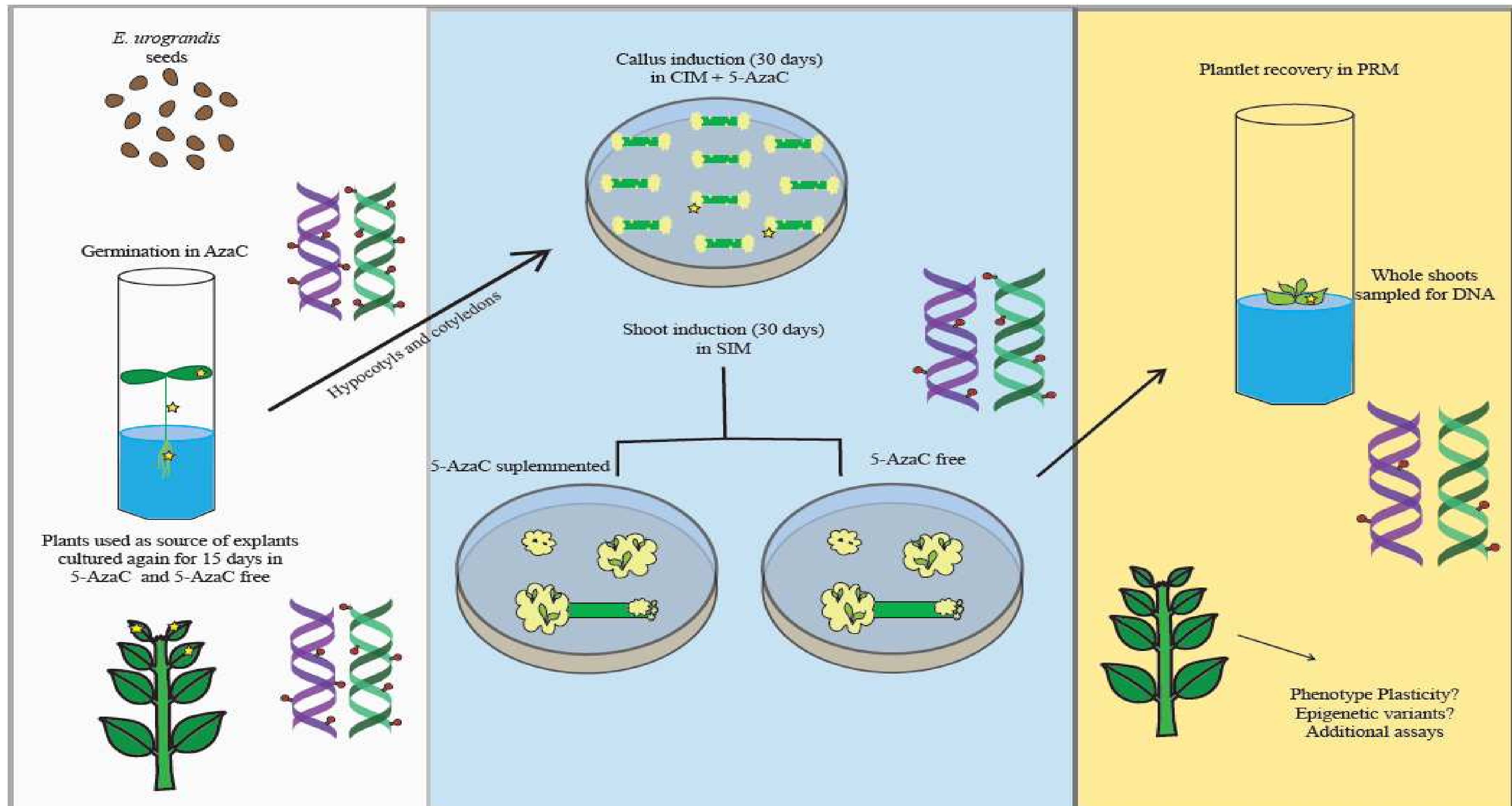

Figure 1: Experimental design for *E. urograndis* demethylation experiment. Seeds are germinated in 5-AzaC, hypocotyls and cotyledons were used as explants for shoot regeneration. Red pins indicate 5-mC levels across the demethylating experiment; Yellow Stars represents the tissue used for epigenetic analysis; CIM = Callus Induction Media, SIM = Shoot Induction Media, PRM = Plantlet Recovery Media. The beige colored board is the recovering step hypometilated plants.

4.4 Results

Germination and rooting under 5-AzaC

After 15 days on germination media, there was a decrease in methylation levels of the explants as increase of 5-AzaC concentrations. Cotyledons had a pronounced effect of demethylation from 1.0 µM to 30 µM, with 5-mC levels ranging from 0.8 to 2.9% when compared to the controls 47.2% (Table 1). Roots otherwise, had an increase at 5.0 µM with 5-mC= 13.2%, whereas 1.0 and 10 µM showed lower levels of 5-mC (3.5 and 2.7%, respectively). Besides, as the concentration of 5-AzaC increase, roots have a slower growth than the controls (Figure 2a). Hypocotyls had different responses, at 5.0 µM there was a hypermethylation of 5-mC= 48%, while 1.4% in 30 µM (Table 1).

As the plants were subcultivated, roots were excised from the first to the second subcultive media. Only two plants demonstrated contrasting phenotypes, with larger leaves and more shoots. In general, plants treated with 5-AzaC had more roots than the control (1.25 roots/plant), with exception of those germinated in 30 and 50 µM and subcultivated in 5-AzaC free media (0.9 and 0.8, respectively). Considering the adventitious roots formation, plants germinated in 1.0 µM and then subcultivated in 5-AzaC free media demonstrated the maximum number of roots 3.6 roots/plant in average (significant at 5% of probability by Tukey's test). Treatments with 1.0 and 5.0 µM had 2.6 roots/plant, and also differed from the controls. Leaves from plants subcultivated in 5-AzaC free media generally presented lower %5-mC levels compared to the controls, and those subsequently cultivated in 5-AzaC (Table 1). Plants germinated in 1.0 µM and 5.0 µM, grown in 5-AzaC free had leaves with lower levels of methylation (1.3% and 0.8 %, respectively).

Table 1 Rates (%) of 5-mC levels under 5-AzaC concentrations [µM] from different explants of 15-days old germinated plantlets, and leaves of subcultivated plants for 30 days (previously germinated in 5-AzaC).

Germinated plantlets				Plants subcultivated			
	Cotyledons	Roots	Hypocotyls	Germination + 5-AzaC*		Germination + 5-AzaC free**	
Controls	47.2	-	-	Controls	3.5	Controls	3.5
0.5	56.5	-	-	0.5 – 0.5	1.0	0.5 – ø	2.5
1.0	0.8	3.5***	-	1.0 – 1.0	3.5	1.0 – ø	1.3***
5.0	1.1	13.2	48	5.0 – 5.0	3.9	5.0 – ø	0.8
10	1.8	2.7	-	10 – 10	1.9	10 – ø	1.2
30	2.9	-	1.4	30 – 30	2.9	30 – ø	2.4
50	-	-	-	50 – 50	1.3	50 – ø	3.6

* Plants were germinated in 5-AzaC and then subcultivated in the same concentrations. ** Plants were germinated in 5-AzaC and subcultivated in 5-AzaC free media. *** Significantly different for adventitious root formation at 5% probability by Tukey's test.

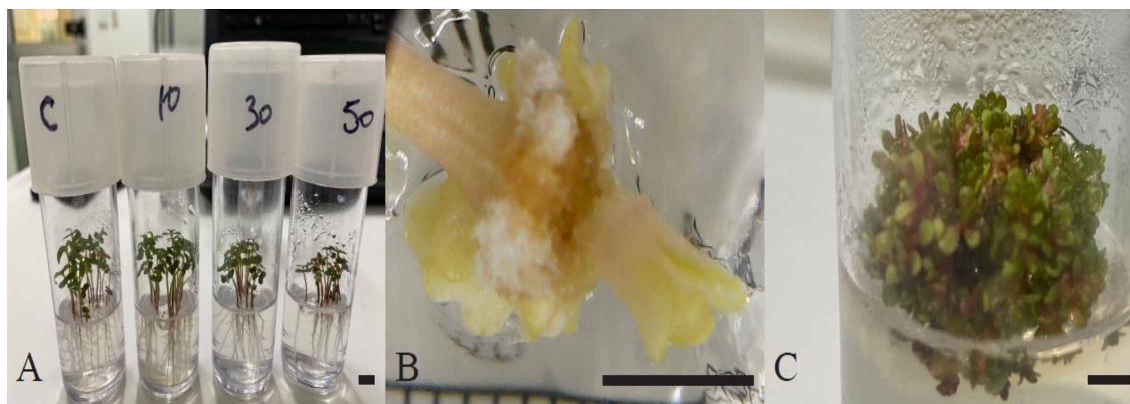

Figure 2: Plantlets germinated in 5-AzaC. A) Root intensity in germination media (from the left to the right, controls, 10 µM, 30 µM and 50 µM); B) Shoots formed in CIM media in the dark, explants were treated with 5-AzaC at 10 µM; C) High intensity of plantlets formed in SIM and transferred to PRM (treatment: 1 µM 5-AzaC). Bars = 0.5 cm.

Subsequent DNA demethylation using 5-AzaC in organogenesis system

After 15 days in CIM culture, callus formation was observed in all treatments of hypocotyls and cotyledons. At the end of 30 days, hypocotyls and cotyledons demonstrated higher rates of callus formation (99 and 100% of the explants). Nevertheless, at this first step of dedifferentiation in the dark, few shoots were observed in both explant types (Figure 2b). Hypocotyls demonstrated shoot formation of 10% for controls and decreasing as the increase of 5-AzaC, 8 and 9% of shoots for 30 and 50 µM, respectively. Cotyledons in its turn, showed lower rates of shoots 3 to 4% regardless of the treatments (Figure 3a,b). The same pattern of shoots formation followed the oxidation rates, higher in hypocotyls as increasing the 5-AzaC (controls= 11.5% and 16.5% at 50 µM), and 3 to 4% of oxidation for cotyledons, independently of 5-AzaC concentrations.

5-mC levels of callus masses at 30 days in CIM indicated an hypermethylation condition of 5-mC= 23.5% for hypocotyls treated with 5.0 µM of 5-AzaC (compared with

other concentrations), and 5-mC= 50.6% for cotyledons when compared to the controls (5-mC= 43.7 %) (Table 2). For cotyledons explants in particular, higher concentrations were able to promote an efficient demethylation to 4% (30 µM) and 2.6% (50 µM).

The SIM media promoted the formation and proliferation of shoots using both explant types. Considering explant responsivity (those capable of produce shoots), hypocotyls explants treated with 1.0 µM of 5-AzaC in CIM and later in 5-AzaC free SIM produced 3.5 responsive explants (forming shoots) being significant at 5% of probability by Tukey's test. However, this treatment resulted in 5 shoots in average, while considering the number of shoots (basic aim of any organogenic protocol), hypocotyls treated with 5.0 µM 5-AzaC during CIM and 5-Azac free in SIM, resulted in average of 6.25 shoots (significant at 5%). For cotyledons explants, the significant treatment for both responsiveness and shoots were at 10 µM of 5-AzaC, generating in average 9.5 responsive explants and 15.5 shoots (significant at 5%). The controls had 6.75 and 6 responsive explants, and 14 and 13 shoots for hypocotyls and cotyledons explants.

Besides counting the number of shoots generated through organogenesis, very often its number was underestimated due to high proliferation capacity of some explants (Figure 2c). The PRM was feasible to elongate and still develop the remaining proliferation of the shoots. A total of 925 shoots were yielded with hypocotyls and 968 shoots from cotyledons (Figure 4a,b). The concentration of 5.0 µM of 5-AzaC in hypocotyls yielded the highest number of regenerated plants 227 from 25 responsive explants (a relation of 9.1 shoots/explant), the same result was found in the controls 213 plant from 24 explants (relation of 8.9 shoots/explant). Cotyledons treated with 1.0 µM of 5-AzaC yielded the best relation of shoots/explant (26.1:1), regenerating a total of 183 plants. Despite of the concentration of 10 µM yielding more plants (189 plants), its relation was around 5.1 shoots/explant, even lower than the controls (6.1:1).

From an epigenetic perspective, hypocotyls plants regenerated showed and hypomethylation condition (Table 2). From the whole experiment, with hypocotyls explants was possible to demonstrate the ability of DNA demethylation through 5-AzaC. Using 5.0 µM, the donor explants showed 48% of 5-mC, later callus masses decreased methylation to 23.5% and the regenerants at the same concentration presented 15.3% 5-mC (Table 1, Table 2). In this treatment, regenerants had half of the methylation levels of the controls (5-mC = 33.1%), and also achieved a higher number of plants.

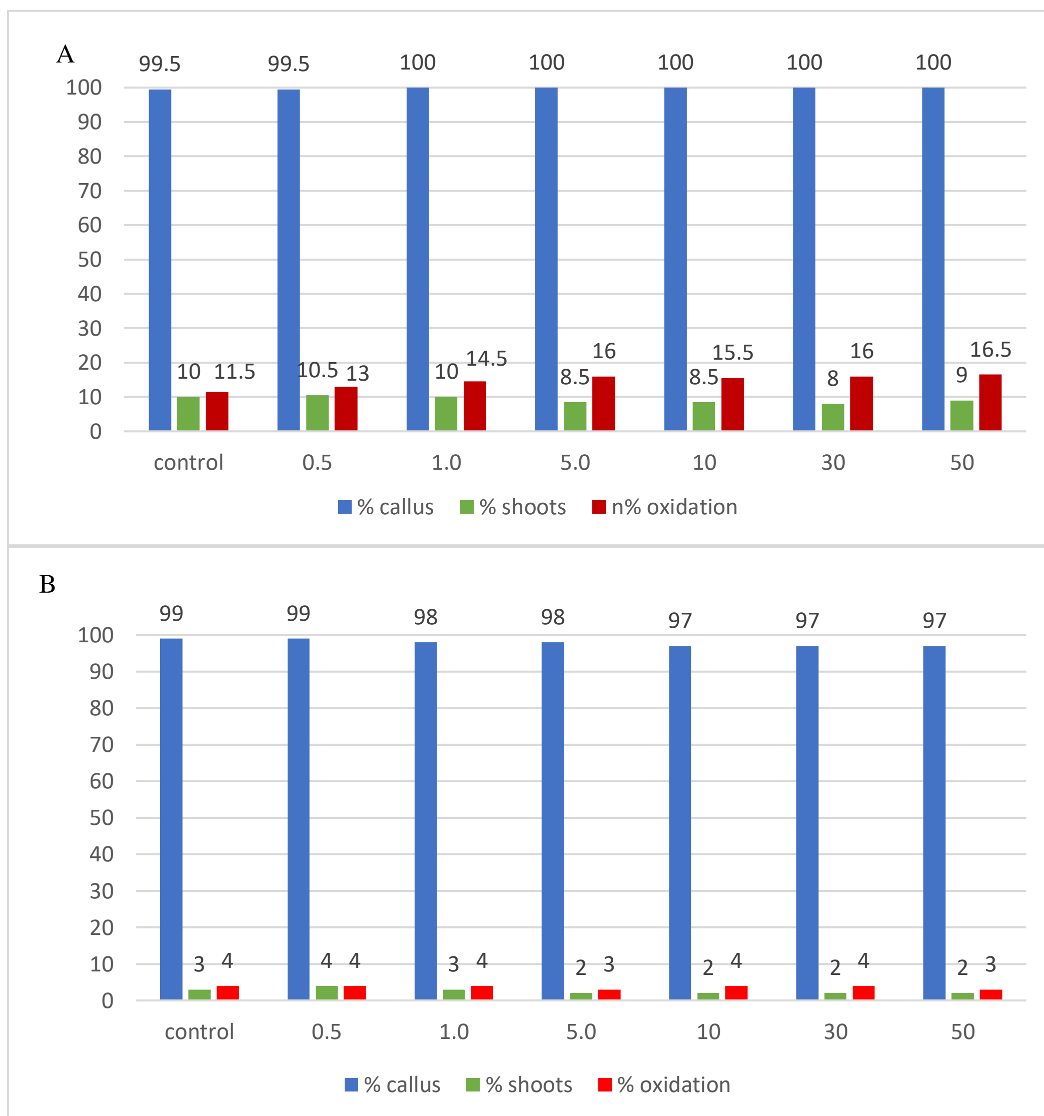

Figure 3: Callus formation from 5-AzaC treatments and controls. A) rates (%) of responsive callus, shoots and oxidation from hypocotyls explants after 30 days in CIM; B) rates (%) of responsive callus, shoots and oxidation from cotyledons explants after 30 days in CIM;

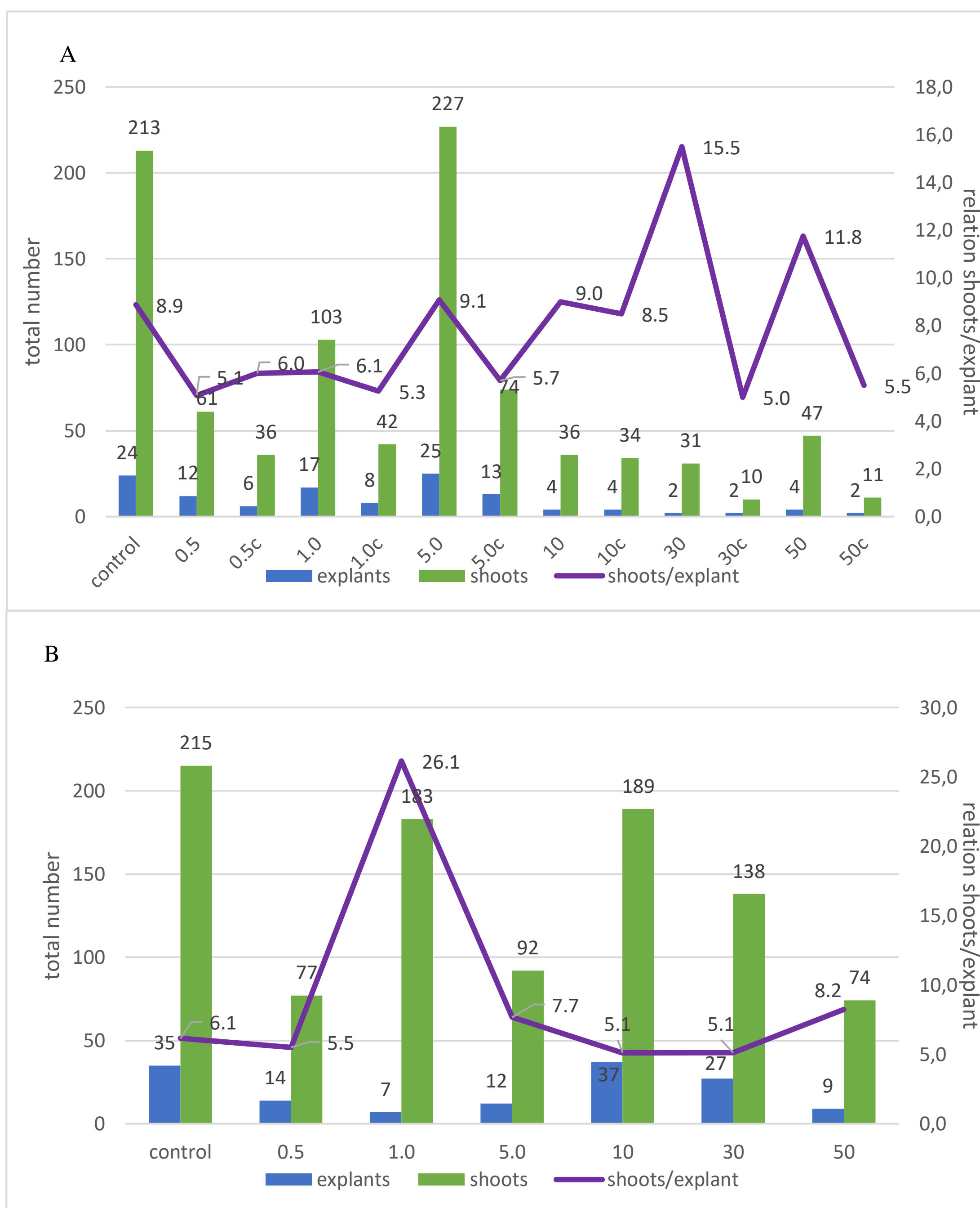

Figure 4: Plantlets recovered from 5-AzaC treatments. A) Total number of plantlets regenerated from hypocotyls explants in SIM media supplemented with 5-AzaC and 5-AzaC free (represented by the letter "c" after each concentration), and relation of number of shoots/exaplant. B) Total number of plantlets regenerated from cotyledons explants in SIM media supplemented with 5-AzaC, and relation of number of shoots/explant.

Table 2 Rates (%) of 5-mC levels under 5-AzaC concentrations [µM] in organogenesis system. %5-mC of callus masses 30-days old in CIM and regenerated plants from hypocotyls explants in PRM.

	Callus mass		Regenerated plants
	Hypocotyls	Cotyledons	Whole Plant*
controls	-	43.7	33.1
0.5	0.2	-	4.8
1.0	3.5	3.8	25.9
5.0	23.5	50.6	15.3
10	-	49.4	-
30	-	4	-
50	3.3	2.6	-

* %5-mC related to the stem and leaves, roots were not formed in this step and therefore not measured.

4.5 Discussion

Effect of 5-AzaC on germination and potential applications in rooting

Aiming the applications of epigenetics in plant breeding, its manipulation must be on tissues that are in constant multiplication, therefore such changes can be transmitted to neighbor cells, tissues and organs as well. Seeds are a good starting material due to its well-coordinated cell division during the seed germination (Massoumi et al., 2017). Hence, the use of demethylation agents such 5-AzaC can be applied in tissue culture media in order to obtain plantlets with a modified epigenetic status, in this case, 5-mC levels. In our work, 5-AzaC on germination of *E. urograndis* seeds promoted a demethylation in whole plantlets. Since epigenetic can be different from tissue to tissue, the sampling method (roots, hypocotyls and cotyledons) allowed to understand how different *Eucalyptus* tissues undergo

the 5-AzaC effect (Table 1). Although demethylation occurred in exposition of 5-AzaC, few treatments demonstrated an hypermethylation profile, such as 5-mC= 56.5 % in cotyledons (0.5 µM), 48% for hypocotyls (5.0 µM) and leaves of subcultivated plants 3.9% (5.0 µM). These higher methylation levels may also occur at lower concentrations of 5-AzaC during somatic embryogenesis of *Cocus nucifera* L. (Osorio-Montalvo et al., 2020), demonstrating that demethylation is not necessarily proportional to the increase in 5-AzaC concentration, but related intrinsic and extrinsic explant conditions.

Another effect was the improvement in rooting, where *E. urograndis* plantlets germinated in 5-AzaC produced more roots than the controls. Moreover, using 1.0 µM on germination and then subcultivated in 5-Azac free media promoted an increase in 65% rooting. In non-juvenile tissues of *Arabidopsis thaliana* L., adventitious root formation was enhanced in 50% using 10 µM 5-AzaC + 30 µM indole acetic acid, as well as when germinated and treated again with 5-AzaC (Massoumi et al., 2017). In fact, there is a correlation with increased DNA methylation and reduction in gene expression, resulting in differences of responsiveness (Saze et al., 2012). In particular for *Eucalyptus* clones, loss of rooting ability is a dramatic problem in clonal forestry resulted by the long-term cycles of vegetative propagation. Thus, 5-AzaC can be used strategically to reduce global DNA methylation and restore rooting ability of elite-clones.

Subsequent DNA demethylation: adding an extra layer of complexity

An organogenesis protocol based on leaves was proposed by França Bettencourt et al. (2020) for efficiently regenerate *E. urograndis* clones. Testing several media, these authors found higher rates of callus formation 71.2% using WPM supplemented with 2.5 µM BAP + 0.5 µM NAA. Since hypocotyls and cotyledons are more juvenile tissues than

leaves, our results presented at least 99% of callus formation for both explants in controls and treatments with 5-AzaC. Such responsiveness and the presence of shoot formation around 8 to 10% at an early stage may be related to the age and methylation levels of these explants.

Since organogenesis is marked by extensive epigenetic changes, the use of 5-AzaC in CIM media was strategic to promote an efficient demethylation in *E. urograndis* explants. In *Populus trichicarpa* Torr & A. Gray, changes in methylome are induced during the *in vitro* callus and regenerated internodes, mainly a 5-mC enrichment of transposable elements (Vining et al., 2013). For such reason we were reluctant to use fresh CIM media each 15 days supplemented with 5-AzaC, otherwise demethylation could inhibit shoot formation through excessive activation of transposable elements.

Improvement of in vitro plant fitness after treatment with 5-AzaC has also been reported to other species in tissue culture. For *A. thaliana,* 50 µM promoted an increase in 50% of rooting formation (Massoumi et al., 2017), while 50 - 200 µM restored the transgenic expression in *Betula platyphylla* Suk. (Zeng et al., 2010). Perhaps the most contrasting effect of 5-AzaC was reported by Munsamy et al. (2013) in sugarcane, using 100 µM + 2,4-D identified plants tolerant to smut (*Sporisorium scitamineum* M. Stoll & Oberw.) and potentially tolerant to the herbicide Imazapyr. Besides the rooting improvement, in our work we identified an increase in shooting for hypocotyls and cotyledons treated with 5 µM and 10 µM of 5-AzaC, respectively. In these treatments was possible to achieve more plants than the controls and more shoots/explant in average (hypocotyls = 9.1, cotyledons = 26.1) and even when compared to basal protocol for *E. urograndis* (França Bettencourt et al., 2020).

Besides its potential, 5-AzaC may also display controversial effects, like accelerate and inhibit growth and development of *Jatropha curcas* L. (Kanchanaketu and Hongtrakul, 2015) or modifying phenotypic traits like flowering time, plant height, branches and biomass

(not always positively in breeding) in *A. thaliana* (Bossdorf et al., 2010), or reducing inducible defenses in *Trifolium repens* L. being more vulnerable to herbivory (Latzel et al., 2020). We observed adverse effect in higher concentrations 30 and 50 µM of 5-AzaC, demonstrated as increase in oxidation rates (%) at CIM media and lower plantlet yield (SIM e RPM media). Such observations can be related as a dosage effect, where 5-AzaC may become cytotoxic at higher concentration (Munsamy et al., 2013). Furthermore, 5-AzaC decrease global DNA methylation but not in a specific way, altering the promoter region of specific genes or rather on the gene "body", activating transposable elements and silenced genes or even increasing methylation in such portions (Bossdorf et al., 2010; Smulders and de Klerk, 2011).

4.6 Conclusions

Our results reinforce that epigenetic changes, mainly global DNA methylation, could improve desirable traits in *E. urograndis*, such as rooting and shooting – two steps with a huge impact in forest chain of pulp and paper industry. Moreover, this was a prospection study whereas the basic material amplifies the phenotype diversity across the *Eucalyptus* germplasm. Further research will be focused on the characterization of these hypomethylated materials, using gene expression profiles combined with metabisulfite sequencing for identification of specific demethylated sites through the genome and also possible somaclonal variations. More detailed insights will clarify our knowledge of phenotype plasticity of elite clones and even whether engineer methylation sites with sophisticated approaches such as with CRISPR/Cas system.

4.7 References

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5. OVERALL CONCLUSIONS

Artificial chromosome set doubling and DNA methylation changes have a pivotal role for *Eucalyptus benthamii* and *Eucalyptus urograndis* phenotypic variability. CSD effect have a huge impact secondary metabolism, more evident for terpene synthesis where autotetraploid presented different chemical compounds not previously found in diploids. Genetic modifications always raise questions around the impact of these plants in the environment. In the present work, we demonstrated that *E. benthamii* autotetraploids are less effective than diploid for larvicidal activity. Moreover, the influence of the essential oils over the shoot growth of *L. sativa* had no difference between the diploid and the autotetraploid condition. Therefore, the use of autotetraploids in the conditions tested demonstrated to be harmless to the environment.

Promoting global DNA demethylation improved the root ability and shoot number during the organogenesis system of *E. urograndis*. These findings may improve the current clonal propagation of elite germplasm of *Eucalyptus,* once frequently these materials lose the adventitial root formation. Moreover, the germplasm with different methylation levels remains for further studies and characterization of the role of epigenetic in phenotype plasticity and desirable traits for *Eucalyptus* improvement.