Research, Society and Development, v. 10, n. 11, e242101119646, 2021
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v10i11.19646

Phytotoxic and cyto-genotoxic activity of essential oil from leaf residues of *Eucalyptus urophylla* and the hybrid *E. urophylla* x *E. camaldulensis* on *Lactuca sativa* and *Sorghum bicolor*

Atividade fitotóxica e cito-genotóxica do óleo essencial de resíduos foliares de *Eucalyptus urophylla* e o híbrido *E. urophylla* x *E. camaldulensis* em *Lactuca sativa* e *Sorghum bicolor*

Actividad fitotóxica y citogenotóxica del aceite esencial de residuo de hoja de *Eucalyptus urophylla* y el híbrido *E. urophylla* x *E. camaldulensis* en *Lactuca sativa* y *Sorghum bicolor*

Received: 08/20/2021 | Reviewed: 08/25/2021 | Accept: 08/27/2021 | Published: 08/30/2021

Thammyres de Assis Alves
ORCID: https://orcid.org/0000-0003-1148-4832
Federal University of Espírito Santo, Brazil
E-mail: thammyresalves@gmail.com
Thayllon de Assis Alves
ORCID: https://orcid.org/0000-0003-2478-851X
Federal University of Espírito Santo, Brazil
E-mail: thayllonalves@gmail.com
Maikon Keoma da Cunha Henrique
ORCID: https://orcid.org/0000-0001-6303-2729
Federal University of Espírito Santo, Brazil
E-mail: maikon.henriques@hotmail.com
Ana Flávia Nunes de Paula Azevedo
ORCID: https://orcid.org/0000-0002-2578-0483
Federal Institute of Espírito Santo, Brazil
E-mail: anaflavia.mulinari@gmail.com
José Augusto Macedo Carvalho
ORCID: https://orcid.org/0000-0002-5735-4550
Federal University of Espírito Santo, Brazil
E-mail: guto_tk22@hotmail.com
Patrícia Fontes Pinheiro
ORCID: https://orcid.org/0000-0003-4019-5773
Federal University of Viçosa, Brazil
E-mail: patriciafontespinheiro@yahoo.com.br
Luciano Menini
ORCID: https://orcid.org/0000-0003-3656-5428
Federal Institute of Espírito Santo, Brazil
E-mail: lmenini@ifes.edu.br
Milene Miranda Praça-Fontes
ORCID: https://orcid.org/0000-0001-7738-9518
Federal University of Espírito Santo, Brazil
E-mail: milene.fontes@ufes.br

Abstract

The use of agrochemicals has influenced the increase in agricultural productivity. However, the concern about damage to the human health and to the environment, resulting from the indiscriminate use of pesticides, has increased worldwide. Alternative methods for controlling pests and diseases have been proposed to maintain productivity and quality of life. A possibility is to use compounds produced by the secondary metabolism of plants, such as the essential oils. Some of these substances perform inhibitory or stimulatory activity on the development of other organisms. Plants from the genus *Eucalyptus* have been investigated and were chosen to be studied in the present work, due to the traditional knowledge regarding the potential of the essential oils from some species, as well as, for the need to use residues from their production. Therefore, were determined the yield, identified the compounds from the essential oils of *E. urophylla* and the hybrid *E. urophylla* x *E. camaldulensis*, and evaluated their biological activity through bioassays, investigating the phyto-cyto-genotoxicity and mutagenicity using *Lactuca sativa* and *Sorghum bicolor* as model plants. The essential oil from the hybrid provided a higher yield. Eucalyptol was the major compound identified for both oils, representing more than 85% of the compounds present. Both *E. urophylla* and the hybrid showed phyto-cyto-genotoxic, mutagenic effects, and clastogenic and aneugenic mechanisms of action, promoting epigenetic changes in the meristematic cells of *L. sativa*. The results point out to the bioherbicidal potential of these essential oils.

Keywords: Aneugenic; Clastogenic; Eucalyptol; Mitotic index; Phyto-cyto-genotoxicity.

Resumo

O uso de agroquímicos tem influenciado o aumento da produtividade agrícola. No entanto, a preocupação com os danos à saúde humana e ao meio ambiente, decorrentes do uso indiscriminado de agrotóxicos, tem aumentado em todo o mundo. Métodos alternativos de controle de pragas e doenças têm sido propostos para manter a produtividade e a qualidade de vida. Uma possibilidade é utilizar compostos produzidos pelo metabolismo secundário das plantas, como os óleos essenciais. Algumas dessas substâncias exercem atividade inibitória ou estimuladora do desenvolvimento de outros organismos. Plantas do gênero *Eucalyptus* foram investigadas e escolhidas para serem estudadas no presente trabalho, devido ao conhecimento tradicional quanto ao potencial dos óleos essenciais de algumas espécies, bem como, pela necessidade de aproveitamento de resíduos de sua produção. Portanto, foram determinados os rendimentos, identificados os compostos provenientes dos óleos essenciais de *E. urophylla* e do híbrido *E. urophylla* x *E. camaldulensis*, e avaliada sua atividade biológica através de bioensaios, investigando a fito-citogenotoxicidade e mutagenicidade utilizando *Lactuca sativa* e *Sorghum bicolor* como planta modelo. O óleo essencial do híbrido proporcionou maior rendimento. O eucaliptol foi o principal composto identificado para ambos os óleos, representando mais de 85% dos compostos presentes. Tanto *E. urophylla* quanto o híbrido apresentaram efeitos fito-citogenotóxicos, mutagênicos e mecanismos de ação clastogênicos e aneugênicos, promovendo alterações epigenéticas nas células meristemáticas de *L. sativa*. Os resultados apontam para o potencial bioherbicida desses óleos essenciais.

Palavras-chave: Aneugênico; Clastogênico; Eucaliptol; Índice mitótico; Fito-citogenotoxicidade.

Resumen

El uso de agroquímicos ha influido en el aumento de la productividad agrícola. Sin embargo, la preocupación por los daños a la salud humana y al medio ambiente, resultantes del uso indiscriminado de plaguicidas, ha aumentado en todo el mundo. Se han propuesto métodos alternativos de control de plagas y enfermedades para mantener la productividad y la calidad de vida. Una posibilidad es utilizar compuestos producidos por el metabolismo secundario de las plantas, como los aceites esenciales. Algunas de estas sustancias ejercen una actividad inhibidora o estimulante del desarrollo en otros organismos. Se investigaron y seleccionaron plantas del género Eucalyptus para ser estudiadas en este trabajo, debido al conocimiento tradicional sobre el potencial de los aceites esenciales de algunas especies, así como a la necesidad de utilizar residuos de su producción. Por lo tanto, se determinaron los rendimientos, se identificaron los compuestos de los aceites esenciales de *E. urophylla* y el híbrido *E. urophylla* x *E. camaldulensis*, y se evaluó su actividad biológica mediante bioensayos, investigando la fitocitogenotoxicidad y mutagenicidad utilizando *Lactuca sativa* y *Sorghum bicolor* como planta modelo. El aceite esencial híbrido proporcionó el mayor rendimiento. El eucaliptol fue el principal compuesto identificado para ambos aceites, representando más del 85% de los compuestos presentes. Tanto *E. urophylla* como el híbrido mostraron efectos fitocitogenotóxicos, mutagénicos y mecanismos de acción clastogénicos y aneugénicos, promoviendo alteraciones epigenéticas en células meristemáticas de *L. sativa*. Los resultados apuntan al potencial bioherbicida de estos aceites esenciales.

Palabras clave: Aneugénico; Clastogénico; Eucaliptol; Índice mitótico; Fito-citogenotoxicidad.

1. Introduction

Technological advances have led to high agricultural productivity, and to lower production costs (Alves and Tedesco, 2015). However, the technologies achieved have generated discussions about their consequences for human health and to the environment (Aragão et al., 2015; Alves et al., 2018; Pinheiro et al., 2015).

Debates have been held regarding the contamination of rivers, groundwater, soils, surrounding cultures, lakes and air (Flores et al., 2004; Silva et al., 2012). We must also consider problems with the aquatic and terrestrial fauna (Flores et al., 2004; Silva et al., 2012). Several cases of intoxication and damage to human health resulting from contact with agrochemicals (either by ingestion or skin contact), have generated the dilemma regarding the benefits and harms of these substances (Carson, 2002; Hendges et al., 2019; Santos et al., 2019).

To maintain an elevated and outstanding productivity and to mitigate the harmful effects of agrochemicals, natural products with biological activity have been evaluated in studies that aim at producing bio agrochemicals. The essential oils from plants with medicinal effects and/or food use are detached among several prominent compounds in this type of research (Aragão et al., 2015; Pinheiro et al., 2015; Vasconcelos et al., 2019).

Species from the genus *Eucalyptus* are known for their essential oils, which serve as the basis of medicinal solutions with anti-inflammatory and antibacterial action and in the treatment of respiratory diseases (Mallard et al., 2018). Studies have confirmed antimicrobial (Dhaliwal et al., 2004; Falahati et al., 2005; Moreira et al., 2005; Oluma and Garba, 2004), insecticide (Erler et al., 2006), antibacterial (Ramezani et al., 2002), phytotoxic and cytotoxic (Aragão et al., 2015) activities. Within this genus, the species *E. urophylla* and the hybrid *E. urophylla* x *E. camaldulensis* are cultivated on a large scale aiming (with their stem) at meeting the needs of the pulp, coal and wood industries (Oliveira, 1998). However, their roots and leaves are residues that are discarded in the production process and there is no study reporting the influence of chemical composition and the action of essential oils of these species on model plants.

Another important fact concerns the planting of eucalyptus trees in India, which are cultivated as part of the local government's social forestry program. These trees are planted on abandoned lands and on the margins of state and national roads. However, dry leaves fall and, depending on the number of individuals found in a certain area, especially in the dry period of the year, they can cause forest fires (Aich et al., 2019). The use of leaf residues from eucalyptus trees for the extraction of essential oils can both increase their economic value and reduce the risk of forest fires.

Researchers have been carrying out bioassays to assess the biological activity, as well as, to elucidate bioactive compounds (Aragão et al., 2015; Alves et al., 2018; Pinheiro et al., 2015). The phyto-cyto-genotoxicity and mutagenicity analysis, besides the determination of mechanisms of action are types of tests performed to determine the herbicidal potential of the studied compounds. These tests present clear and efficient results in a short period of time, use model organisms (both mono and eudicotyledonous), present low cost and use, as test organisms, plants that show results correlated with those found in mammals (Carvalho et al., 2019; dos Santos et al., 2018; Silveira et al., 2017).

Given the above, the objective was to evaluate the chemical composition and the biological activity of the essential oils of *E. urophylla* and the hybrid *E. urophylla* x *E. camaldulensis*. The tests were carried out with the plant models *Lactuca sativa* L. and *Sorghum bicolor* (L.) Moench, through phytotoxicity and cytotoxicity assessments. Also assessed the similarity of the variables between the two species, since studied a hybrid and its parent.

2. Methodology

2.1 Plant Material

Leaves from adult clones of *E. urophylla* (parental) and the hybrid *E. urophylla* X *E. camaldulensis* were harvested in the forest garden of the Center of Agricultural Sciences and Engineering of the Federal University of Espírito Santo, located at Highway ES 482, Km 43, coordinates 20°47'43.7"S 41°24'20.9"W. For the biological effect analysis, seeds of *L. sativa* (lettuce) “Crespa Grand Rapids - TBR” (ISLA) and *S. bicolor* (sorghum) “precious AL” (Br seeds) were purchased from local stores.

2.2 Essential Oil

For the oil extraction, the leaves were weighed, crushed with distilled water and placed into the Clevenger apparatus. Subsequently, the oil obtained was weighed and its compounds were identified by gas chromatography with a mass spectrometer (Mendes et al., 2018). Helium was used as a carrier gas in a capillary column of fused silica Rtx-5MS (30 m long, 0.25 mm internal diameter). The temperatures were 220°C for the injector and 300°C for the detector, being the 60°C the initial column temperature, programmed for an increase of 3°C per minute until reaching 240°C, the maximum temperature. The obtained mass spectra was compared with reference data from the equipment database. This procedure was performed separately for the two studied species.

For component quantification, the essential oils were analyzed on a gas chromatograph equipped with a flame ionization detector (GC-FID), using nitrogen as carrier gas a the stationary phase was the capillary column Rtx-5MS (30 m long and 0.25 mm internal diameter).

The yield of each essential oil was expressed in percentage. It was calculated through the comparison of the quantity (g) of leaves used to obtain the oil with the quantity (g) of oil obtained.

2.3 Phytotoxicity Assay

To perform the phytotoxicity test, five concentrations (3000, 1500, 750, 375 and 187.5 μg L^{-1}) of each essential oil obtained were analyzed. A completely randomized design was used with four replications per treatment; each replication consisted of 25 seeds. The analyses were carried out with lettuce and sorghum. Distilled water and dichloromethane (solvent used in the preparation of the concentrates) were used as negative controls (C-), and glyphosate, commercial herbicide, was used as a positive control (C+), at the concentration indicated by the manufacturer, 0.1%.

Evaluated the germination speed index (GSI) of the seeds following the number of seeds germinated per plate every 8 hours during the first 48 hours of exposure to the treatments. The germination percentage (GP) and the percentage of oxidized roots (O) were evaluated based on the germinated seeds/oxidized roots per plate after 48h of exposure to the treatments. Root (RG) and shoot (SG) growths were measured after 48h and 120h of exposure to the treatments, respectively (Aragão et al., 2015).

2.4 Cyto-Genotoxic and Mutagenic Assay

The tests were performed on lettuce roots after 48 hours of exposure to the treatments, with the same experimental design described on item 2.3. Only lettuce was used, since it presents large chromosomes in small quantities, easy manipulation of roots and preparation of slides and because it has a correlation with the response in the cell cycle of other eukaryotes (Silveira et al., 2017).

For the cellular evaluation, roots were collected and fixed in ethyl alcohol:acetic acid (3:1). After 24 hours of fixation, the slides were prepared using 2% of acetic orcein as dye. Approximately 1000 cells were evaluated in each slide. We classified them according to the phase of the mitotic cycle and regarding the cellular damage presented.

To assess cytotoxicity, the mitotic index (MI) was calculated by dividing the number of cells in division per the total number of cells evaluated. The genotoxic effect was assessed based on the amount of observed chromosomal alterations (CA). CA were calculated from the ratio between the number of cells with CA and the total number of cells evaluated, as well as, by the frequencies of each observed CA, which are calculated by the ratio of the quantity of each CA to the total cells in division observed. The mutagenic effect was measured from the nuclear alterations (NA), calculated from the ratio between the number of cells with NA and the total number of cells evaluated, as well as, by the frequencies of each observed NA, calculated using the ratio between the quantity of each NA and the total number of cells evaluated (Aragão et al., 2015).

2.5 Statistical Analysis

The results were subjected to analysis of variance and the means were compared by the Dunnett's test ($p < 0.05$), the most suitable test for comparing treatments with controls. All data were processed with the GENES statistical program (Cruz, 2013).

3. Results and Discussion

3.1 Quantification and Characterization of Essential Oils

The essential oil of the hybrid showed a yield of 0.77% m/m, while *E. urophylla* presented 0.20% m/m per fresh leaf fraction. The hybrids have a high genetic potential for production, because this technique allows exploring heterosis (superiority of the crossing in relation to either two parents), also called hybrid vigor. Cimanga et al. (2002) studied the essential oils of different species of eucalyptus and obtained a yield of 0.53% m/m from the essential oil of *E. urophylla*, using the same extraction method and comparing it with the weight of fresh leaves. The variation observed may be due to environmental conditions, plant and leaf age as well as the genetic factors (Xavier et al., 1993).

Cimanga et al. (2002) also studied the essential oil of *E. camaldulensis*, the other parent of the studied hybrid. The data obtained showed lower yield of this parent (0.30% m/m of yield) when compared to *E. urophylla*, which presented 0.53% m/m. The individuals from the current research were harvested in the same place (same environmental conditions) and followed the same pattern of size and age of individuals and leaves (minimizing the physiological differences). Can infer that the yield of the hybrid (0.77% m/m), because it is higher than that of the parents', results from the additive effect of the combination of parental genes, determining a higher yield in the hybrid oil.

Regarding the chemical characterization, were identified four compounds in each oil (four peaks appeared for each oil). The major compound in the essential oil of *E. urophylla* was the oxygenated monoterpene eucalyptol (87.9%), followed by the oxygenated sesquiterpene viridiflorol (5.1%), the hydrocarbon monoterpene α-pinene (4.3%) and the hydrocarbon sesquiterpene aromadendrene (2.6%) (Table 1). Eucalyptol was also the major compound identified in the hybrid (89.8%), followed by the oxygenated monoterpene terpineol (6.73%), hydrocarbon monoterpene α-pinene (1.9%) and the oxygenated monoterpene linalool (1.4%) (Table 1).

Table 1. Compounds present in the essential oils of *E. urophylla* and the hybrid *E. urophylla* x *E. camaldulensis*.

Species	Compound	Retention time (minutes)	Pico relative area (%)
Eucalyptus urophylla	α-Pinene	8.3	4.3
	Eucalyptol	12.6	87.9*
	Aromadendrene	30.7	2.6
	Viridiflorol	27.1	5.1
Eucalyptus urophylla* X *Eucalyptus camaldulensis	α-Pinene	8.3	1.9
	Eucalyptol	12.6	89.8*
	Linalool	19.9	1.4
	Terpineol	27.1	6.73

* Major compound. Source: Authors.

Eucalyptol has been described in the literature as the major compound of both the essential oils of *E. urophylla* and *E. camaldulensis* (Cimanga et al., 2002; Pereira, 2010), which was also observed in the oil of the hybrid studied in the present research. In addition, the presence of α-pinene, eucalyptol and terpineol in both parents has already been described in the literature, while the presence of linalool was observed only in the parental *E. urophylla* (Cimanga et al., 2002; Pereira, 2010).

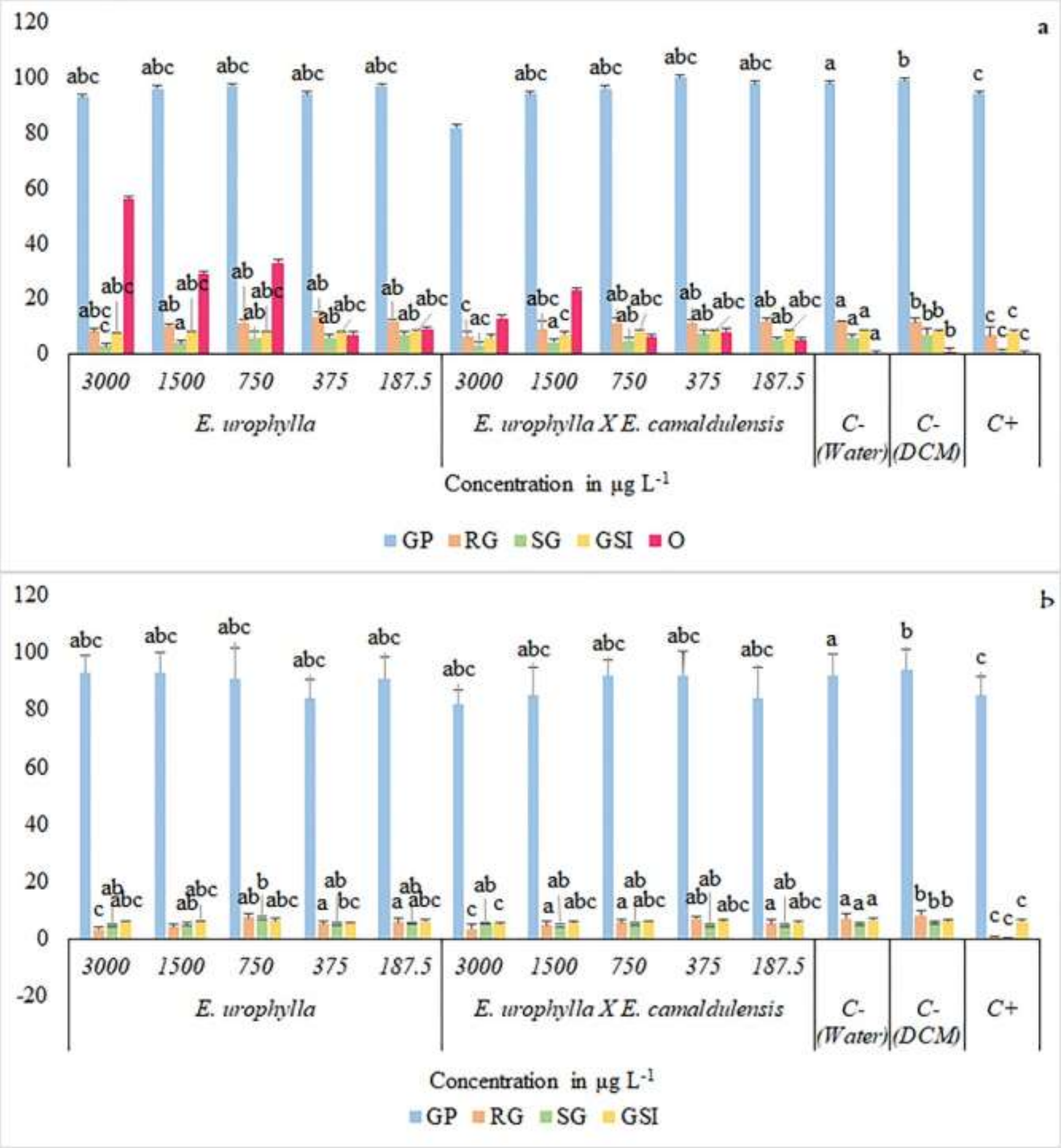
The hydrocarbon monoterpene α-pinene is present in both parents. According to Pereira (2010), this compound represented 1.7% of the oil from *E. camaldulensis* and 5.0% of the oil from *E. urophylla*. Thus, the percentage of α-pinene identified in *E. urophylla* in the present study, is similar to that found by that author. The percentage of α-pinene identified in the hybrid in the present study, is more similar to that found in the parental *E. camaldulensis* by Pereira (2010). Linalool has been described as part of the composition of the oil from *E. urophylla*, representing 2.5% of the total oil composition (Cimanga et al., 2002). Thus, there is a decrease in the proportion observed in the hybrid, when compared to the parental.

Other authors have already reported terpineol as part of the essential oils of the parental species, however, as explained previously, in lower proportions than that found in the hybrid in the present study, indicating the presence of a possible additive effect in its production.

2.2 Phytotoxicity Assay

Phytotoxicity analyses demonstrated the toxic and allelopathic effects of both the tested oils. GP was significantly inhibited, differing even from C+, in lettuce seeds treated with the hybrid oil at a concentration of 3000μg L^{-1}, with a 17% inhibition compared to C- (Figure 1a). This is the low-sensitive parameter, which is only expressed in the acute phytotoxicity of the test compounds (Aragão et al., 2017; Costa et al., 2019). Therefore, as it is not very sensitive, this variable will be changed when the test agent is very toxic, that is, when it produces acute toxicity. Thus, the reduction of this variable demonstrated the strong bioherbicidal potential of the hybrid's essential oil.

Figure 1. Phytotoxicity of the essential oils of *E. urophylla* and of the hybrid *E. urophylla* X *E. camaldulensis* at the concentrations of 3000, 1500, 750, 375 and 187.5µg L^{-1} in (a) lettuce and (b) sorghum. Means followed by letter a were equal to C- (distilled water), means followed by letter b were equal to C- (dichloromethane-DCM) and means followed by letter c were equal to C+ (glyphosate), according to Dunnett's test ($p<0.05$). Where: GP - represents the percentage of seeds germinated after 48 hours of exposure to the treatments; GSI - the seed germination speed index during the first 48h, evaluated every 8h, after exposure to the treatments; O - percentage of oxidized roots after 48 hours of exposure to the treatments (only observed in lettuce); RG - root size (mm) after 48h of exposure to the treatments; SG – shoot size (mm) after 120h of exposure to the treatments.



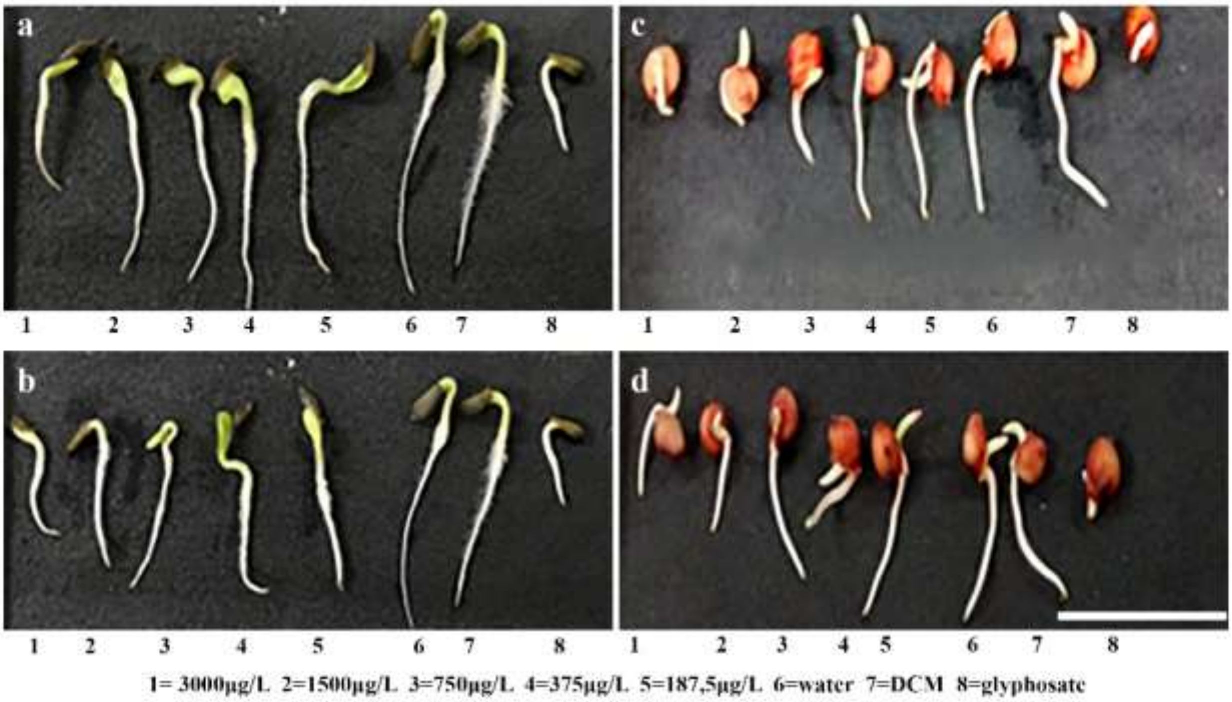
Source: Authors.

All the treatments showed GSI means lower than those of C-, showing statistical difference in the treatments of the hybrid at the concentration of 3000µg L^{-1} in lettuce and sorghum, and at the concentration of 1500 µg L^{-1} in lettuce, equal to C+ (Figure 1). According to Pinheiro et al. (2015), this is the most sensitive parameter among those evaluated in the phytotoxicity tests; its inhibition indicates the toxicity of the test agent regardless of the results observed in the other measured parameters. The delay in the germination period represents a competitive disadvantage for the individuals in the field, as they will be submitted to a longer period of exposure to pathogens, abiotic stress and predators. The prolongation in the germination time determines competitive losses with pre-established plants in growth, making them more susceptible (Uhlmann et al., 2018).

The two oils tested promoted oxidation (O) in the lettuce roots, with no variation in sorghum (Figure 1). All the oil treatments caused an increase in O in lettuce, which was significant in the treatments with the essential oils of *E. urophylla* at the concentrations of 3000, 1500 and 750µg L^{-1}, 56, 29 and 33 times, respectively, when compared with C-, this increase was higher than that promoted by C+ (Figure 1a). This oxidative phenomenon, expressed from the darkening of the roots, has been related to the production, oxidation and release of phenolic compounds, which inhibit the individual's growth and development (Melo et al., 2001).

The hybrid's oil (3000µg L^{-1}) caused significant inhibition in the RG of both model species, comparing to C- and equaling to C+, with 48% in lettuce and 60% in sorghum, when compared with C- (Figure 1 and 2). The essential oil of *Eucalyptus urophylla* inhibited the root development of sorghum by 60% and 50%, at the concentrations of 3000 and 1500µg L^{-1}, respectively, when compared to C- (Figure 1b). This result may be associated with the oxidative toxic effect described above, which promoted a decrease in the RG of the seedlings. Aragão et al. (2017) described this variable as the most sensitive among those related to growth.

Figure 2. Illustration of the different concentrations effects of essential oils of *E. urophylla* (a and c) and with the hybrid *E. urophylla* X *E. camaldulensis* (b and d) on the root growth of lettuce (a and b) and sorghum (c and d) seedlings. Bar = 1 cm.



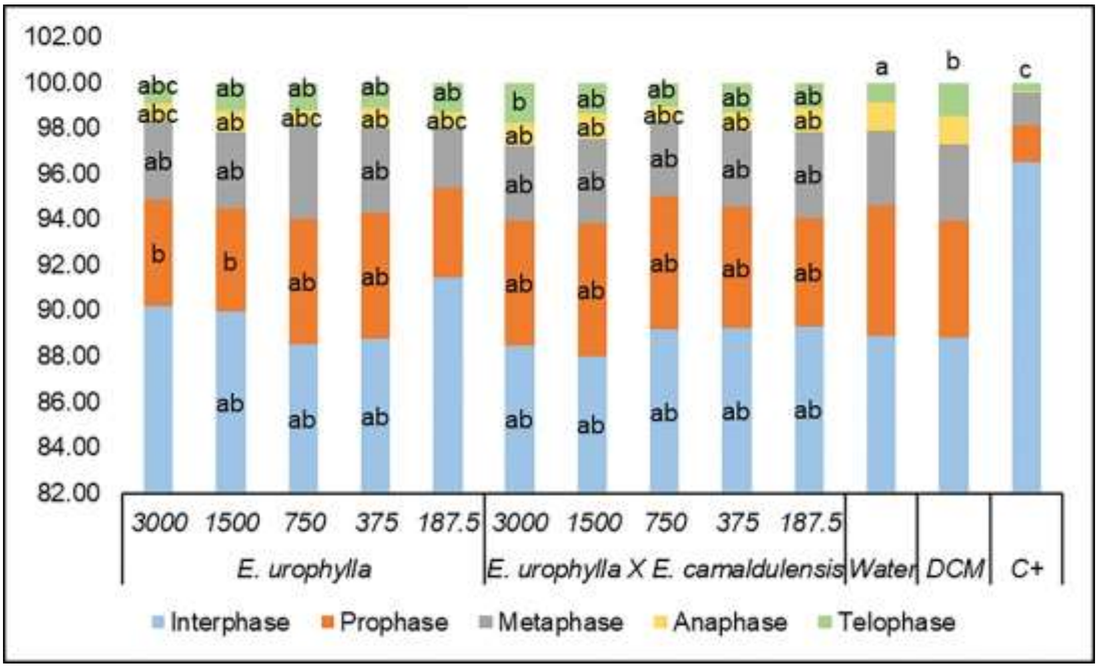
Source: Authors.

The essential oil of the hybrid did not promote significant inhibition in the SG of the model plants (Figure 1). However, the essential oil of *E. urophylla* inhibited lettuce's SG by 60%, at the concentration of 3000 µg L^{-1}, which was equal to C+ (Figure 1a). This result may be related to the chemical composition of the essential oils, as well as to the synergistic effect between the compounds. Aragão et al. (2015), verified the allelopathic activity of *E. grandis* and *E. citriodora*, and reported the synergistic effect of the essential oil of *E. grandis*, which presents α-pinene as its major compound, providing greater phytotoxic effect. When we compare these results with ours, conclude that α-pinene, which is more abundant in *E. urophylla* than in the hybrid, provided greater phytotoxicity, when in synergism with the other compounds of the oil, providing greater inhibition in the SG of seedlings treated with *E. urophylla*, than with its hybrid.

3.3 Cyto-Genotoxic and Mutagenic Assay

The essential oil of *E. urophylla*, promoted a significant increase of 1.5 and 3%, at the concentrations of 3000 and 187.5µg L^{-1}, respectively, in the frequency of cells in interphase (Figure 3). The increase in cells in interphase indicates the occurrence of a mitosis block (Aragão et al., 2015; Barroso Aragão et al., 2017), which may be related to the inhibition of DNA synthesis due to the increased damage to this molecule, aiming at minimizing tissue damage (Kordali et al., 2008). Aragão et al. (2015) also observed this result in treatments with the essential oils of *E. grandis* and *E. citriodora*.

Figure 3. Percentage of each cell cycle phase of lettuce root meristems treated with the essential oils of *E. urophylla* and the hybrid *E. urophylla* X *E. camaldulensis* at the concentrations of 3000, 1500, 750, 375 and 187.5µg L^{-1}. Means followed by letter a were equal to C- (distilled water), means followed by letter b were equal to C- (dichloromethane-DCM) and means followed by letter c were equal to C+ (glyphosate), according to Dunnett's test ($p < 0.05$).

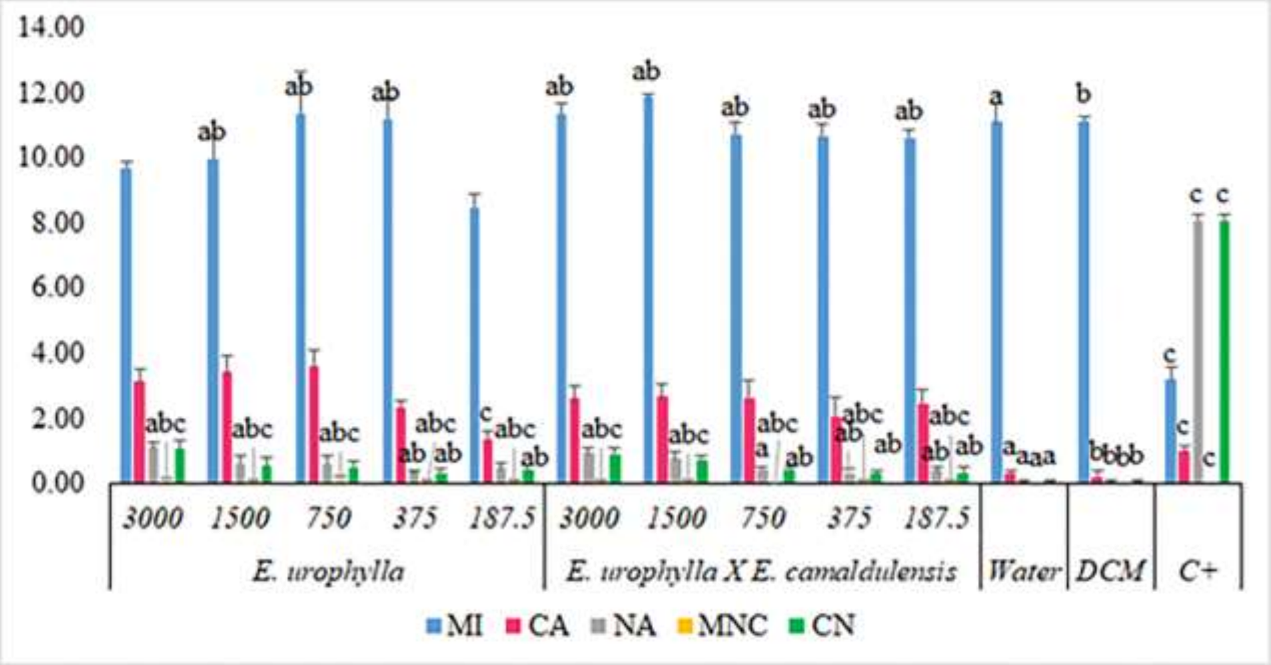


Source: Authors.

The essential oil of *E. urophylla* caused a decrease in the percentage of prophase and metaphase in 31% and 21%, respectively, at the lowest concentration (187.5µg L^{-1}) (Figure 3). This reinforces the previous result and may be related to the increased oxidation of the roots treated with this oil (Figure 1), since this phenomenon is related to the release of phenolic compounds, which are toxic to the plant (Aragão et al., 2017). In addition, the essential oil of *E. urophylla* resulted in an increase of 24% in the frequency of metaphases at the concentration of 750µg L^{-1} (Figure 3). This is related to the increase in chromosomal adherence, which alters the pattern of the phases of mitotic division and determines the cell permanence in metaphase (Aragão et al., 2017; Alves et al., 2018; dos Santos et al., 2018).

The most cytotoxic treatments were those of the essential oil of *E. urophylla* at the concentrations of 3000 and 187.5µg L^{-1}, which significantly inhibited MI by 13 and 23%, respectively (Figure 4). This parameter is a well-established endpoint as an indicator of toxic agents. Under stress conditions, cells will suffer a reduction in the number of cells in division, aiming at minimizing the contact with the toxic substance (Aragão et al., 2015; Aragão et al., 2017; Costa et al., 2017; Alves et al., 2018; Leme and Marin-Morales, 2009; Pinheiro et al., 2015).

Figure 4. Cellular and chromosomal alterations in root meristems of lettuce treated with the essential oils of *E. urophylla* and the hybrid *E. urophylla* X *E. camaldulensis* at the concentrations of 3000, 1500, 750, 375 and 187.5µg L^{-1}. Means followed by letter a were equal to C- (distilled water), means followed by letter b were equal to C- (dichloromethane-DCM) and means followed by letter c were equal to C+ (glyphosate), according to Dunnett's test ($p < 0.05$). The abbreviations represent: MI - mitotic index, CA - chromosomal alterations, NA - nuclear alterations, MNC - micronucleus, CN - condensed nucleus.



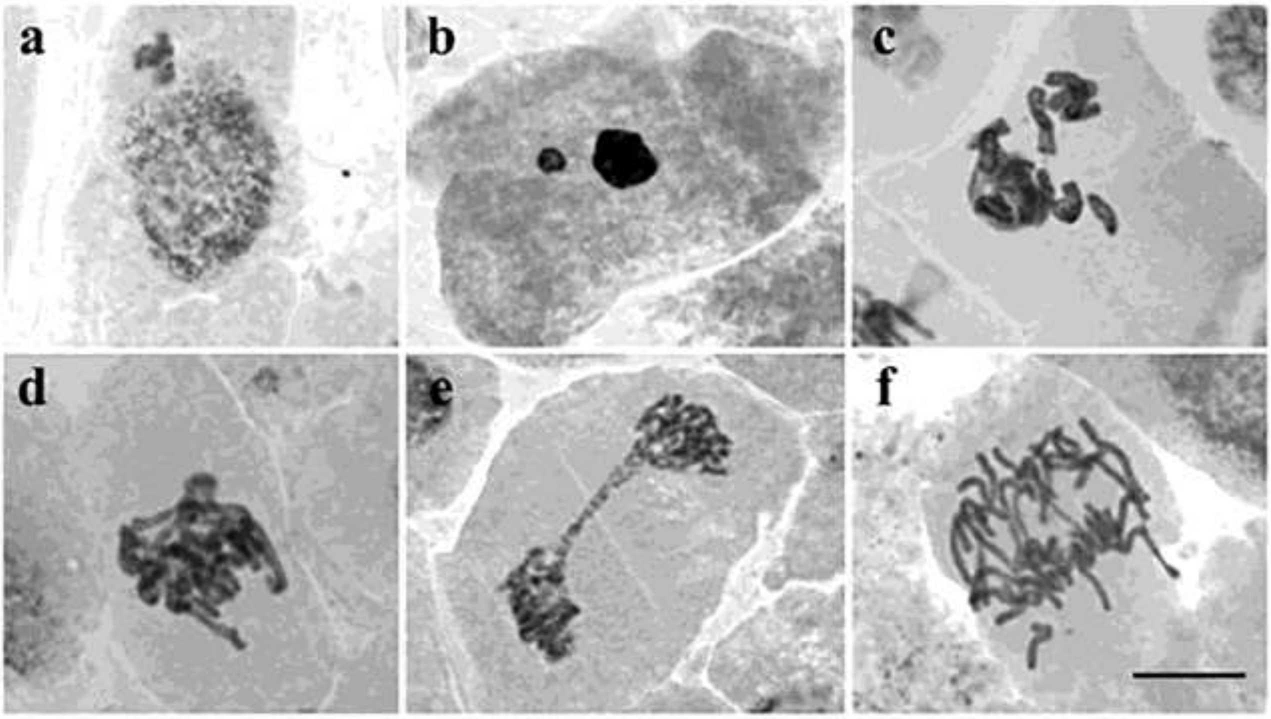
Source: Authors.

All the treatments were genotoxic, promoting a significant increase in CA, when compared to C- and C+. The treatments with *E. urophylla* were increased 9.4; 10.3; 11; 7.96; 4.15 times, at the concentrations of 3000, 1500, 750, 375 and 187.5µg L^{-1}, respectively (Figure 4). For the treatments with the hybrid's oil at the concentrations of 3000, 1500, 750, 375 and 187.5µg L^{-1} this increase was of 7.8; 8; 7.96; 6.27 and 7.15, respectively (Figure 4). CA are caused by the interaction between the genetic material of the cell and the chemical compound present in the environment, what is a genotoxicity endpoint (Bernardes et al., 2015).

The essential oil of *E. urophylla* presents greater mutagenic potential than the hybrid's oil. Both promoted an increase in the frequency of NA, however, the essential oil of *E. urophylla*, promoted a significant increase of 15.7; 8.5; 8.5 and 6.71 times, at the concentrations of 3000, 1500, 750, and 187.5µg L^{-1}, respectively. While the essential oil of the hybrid provided a significant increase of 5.5 and 11 times, at the concentrations of 3000, 1500µg L^{-1}, respectively, when compared to C- (Figure 4). NA are morphological alterations, which occur as a result of biochemical changes in the cell nucleus, as a defense mechanism for possible nuclear "errors" arising from interaction with the environment (by Alves et al., 2018; dos Santos et al., 2018; Fernandes et al., 2009).

The observed NA were micronucleus (MNC) and condensed nucleus (CN) (Figure 5a and 5b). CN was more frequent contributing on a larger scale in the total of NA (Figure 4). The MNC comes from chromosomes that are "lost" during cell division (not properly incorporated into the nucleus of the daughter cell) or it is formed by fragments of acentric chromosomes (also not incorporated into the nucleus of the daughter cells). Thus, these MNCs have the function to involve and eliminate these extra/loose portions of DNA from the cell cytoplasm (dos Santos et al., 2018; Fernandes et al., 2009).

Figure 5. Nuclear alterations, in (a) micronucleus and (b) condensed nucleus; and chromosomal, in (c) c-metaphase, (d) adherent, (e) bridge in telophase, (f) bridge in anaphase with lost chromosome, observed in meristematic cells of lettuce treated with the essential oils of *E. urophylla* and *E. urophylla* X *E. camaldulensis* at the concentrations of 3000, 1500, 750, 375 and 187.5µg L^{-1}.



Source: Authors.

The cells treated with the essential oil of *E. urophylla* at the concentrations of 3000, 1500 and 750µg L^{-1}, presented a frequency of CN significantly higher than the frequency of C-, 14.71, 7.57 and 7.14 times, respectively. Whereas, the hybrid's oil promoted a significant increase of 12.8 and 10.4 times, at the concentrations of 3000 and 1500µg L^{-1}, respectively (Figure 4). CN is the cytological expression of the occurrence of cell death (Andrade-Vieira et al., 2011; Costa et al., 2017). This mechanism has the main function of maintaining tissue homeostasis, in addition to eliminating cells that could trigger malignant processes in the body, as they contain changes in DNA (Silva et al., 2017). This variable is increased according to the increase in genetic damage (Kordali et al., 2008).

The CA observed were: c-metaphase (Figure 5c), chromosomal adhesion (Figure 5d), chromosomal bridge (Figure 5e), missing chromosome (Figure 5f), multipolarity, chromosome delay in telophase and chromosomal fragment. The frequencies of each CA clarify the mechanism of action of the oil, which can be clastogenic or aneugenic (Aragão et al., 2015; Costa et al., 2019; Alves et al., 2018; Fernandes et al., 2009).

The aneugenic mechanism of action is promoted due to changes in the mitotic machinery of the cell, promoting changes in the mitotic spindle, involving the polymerization and depolymerization of the microtubules. Meanwhile, clastogenic agents interact with the genetic material of the cell, causing DNA damage (Bernardes et al., 2015; dos Santos et al., 2018; Fernandes et al., 2009). There was an increase in the frequency of chromosomes lost in all the treatments analyzed, with significance in treatments with the essential oil of E. urophylla at the 3 highest concentrations, 13.9, 10.94 and 13.6 times, respectively, which were equal to C+ (Table 2).

Observed multipolarity, an aneugenic alteration, in cells treated with the essential oil of *E. urophylla* at concentrations of 1500 and 375μg L^{-1}, with no significant increase (Table 2). This CA is related to the aneugenic action, since it results from the formation of multiple nucleation sites, which can be caused by the genetic imbalance of the cell, occurring in polyploid cells (Fernandes et al., 2009).

Chromosomal adherence was the most frequently observed alteration, with an increment in all the treatments. It showed a significant increase of 6.45; 6.94; 7.61 and 6.04 times in the treatments with *E. urophylla* at the concentrations of 3000, 1500, 750 and 375μg L^{-1}, as well as, 7.7; 6.22 and 6.08 times in cells treated with the hybrid's oil at the concentrations of 1500, 750 and 187.5μg L^{-1}, respectively (Table 2). Chromosomal adherence occurs due to cytological, genetic and epigenetic changes. The latter is indicated, since such alterations comes from changes in the phosphorylation pattern of histones, suggesting both aneugenic and clastogenic action (dos Santos et al., 2018; Freitas et al., 2016; Silveira et al., 2017).

C-metaphase was very frequent and presented an increase in all the treatments. Nevertheless, it did not show a statistical difference from C-, due to the low number of cells in C+ division, making the frequency of c-metaphases (which is obtained by the reason of the number of cells with c-metaphase observed by the total number of dividing cells) high in this treatment (Table 2). The c-metaphases demonstrate aneugenic action of the test agent, since they express the complete inactivation of the microtubules and consequently the formation of the mitotic spindle (Carvalho et al., 2019; dos Santos et al., 2018).

Another alteration that presented (non-significant) increase in all the treatments was the delay of chromosomes in telophase (Table 2). This alteration is related to the interference of the essential oils tested in the depolymerization of the microtubules, resulting in an uneven dragging of the chromosomes. Such alteration causes the cell to reconstitute a nuclear envelope with deformation, aiming at involving all the genetic material applicable to it and later that deformation is undone (Fernandes et al., 2009).

The frequency of chromosomal bridge was significantly increased in all of the treatments, 16 times, when compared to C-, in cells treated with the oil of *E. urophylla* at the concentration of 1500μg L^{-1}. A similar fact was observed in chromosomal fragmentation, which had a significant increase in the same treatment (Table 2). The increase in the frequency of bridges and chromosomal fragments indicates the clastogenic action of the test agent (Fernandes et al., 2009). This result suggests the occurrence of the break-fusion-break cycle, where chromosomal fragmentation occurs leaving free cohesive ends in the chromosomes, which causes the fusion (bridge) of chromosomes. When these chromosomes are dragged by their centromeres to the cell poles, through the depolymerization of the alpha and beta tubulin filaments, the chromosome breaks again, forming new chromosomal fragments, enabling new fusion and future breaks (Silveira et al., 2017).

Elucidative experiments encompassing phyto-cyto-genotoxicity, mutagenicity and the mechanism of action of natural compounds is important to elucidate the best use of plant material/waste, as well as to minimize environmental damage resulting from forest fires.

Table 2. Chromosome alterations observed in meristematic cells of lettuce treated with essential oils of *E. urophylla* and of the hybrid at the concentrations of 3000, 1500, 750, 375 and 187.5μg L^{-1}.

	μg L^{-1}	Lost	Multipolar	Adherent	C-Met	Bridge	Delay	Fragment
E. urophylla	3000	8.23c	0.00abc	9.62c	5.16ab	3.78abc	5.49ac	0.00 abc
	1500	6.46c	0.65abc	10.35c	4.96ab	5.02c	5.32abc	1.70
	750	8.07c	0.00abc	11.35	4.36ab	3.27abc	4.04abc	0.78abc
	375	2.30abc	0.57abc	9.01c	1.75ab	3.28abc	3.55abc	0.60abc
	187.5	1.18abc	0.00abc	7.44ac	2.34ab	2.02abc	2.80abc	0.41abc
E. urophylla X *E. camaldulensis*	3000	3.57abc	0.00abc	4.39abc	6.45ab	4.07abc	3.80abc	0.60abc
	1500	1.97abc	0.00abc	11.48	1.41ab	3.35abc	3.90abc	0.28abc
	750	4.93abc	0.00abc	9.28c	3.08ab	2.16abc	4.94abc	0.00abc
	375	2.79abc	0.00abc	7.22ac	1.58ab	3.43abc	3.79abc	0.62abc
	187.5	4.36abc	0.00abc	9.06c	3.10ab	4.69abc	1.56abc	0.32abc
water		0.59a	0.00a	1.49a	0.00a	0.31a	0.62a	0.00a
DCM		0.30b	0.00b	0.90b	0.00b	0.30b	0.30b	0.00b
C+		5.17c	0.00c	4.52c	18.20c	1.19c	2.02c	0.00c

Means followed by letter a were equal to C- distilled water, means followed by letter b were equal to C- dichloromethane (DCM) and means followed by letter c were equal to C+ glyphosate, according to Dunnett's test ($p < 0.05$). Source: Authors.

4. Conclusion

The yield and characterization of the essential oils of *E. urophylla* and its hybrid *E. urophylla* X *E. camaldulensis* indicated a possible additive effect of the inherited genes for such characteristics. The yield of the essential oil of the hybrid was superior to that of the parents.

Both *E. urophylla* and the hybrid showed phyto-cyto-genotoxic and mutagenic activities, as well as clastogenic and aneugenic mechanisms of action. In addition, they promoted epigenetic changes in the meristematic cells of lettuce, identified from the increase in chromosomal adherence. This was the first report of the action mechanism of essential oils of the *E. urophylla* and *E. urophylla* X *E. camaldulensis* in model plant cells.

The results point out to the bioherbicidal potential of these oils, raising the possibility that the leaf residues of these species are destined for the extraction of essential oil, aiming at its application in the control of weeds.

Acknowledgments

The authors thank the Foundation for Research and Innovation Support of Espírito Santo (Fapes), the National Council of Scientific and Technological Development (CNPq). This study was funded in part by the Coordination of Improvement of Higher Level Personnel - Brasil (CAPES) - Finance Code 001.

References

Aich, S., Nandi, B. K. & Bhattacharya, S. (2019). Effects of eucalyptus leaves blending on combustion characteristics of an Indian reject coal. *Energy Source Part A*, 1-12. https://doi.org/10.1080/15567036.2019.1644397

Alves, C. T. & Tedesco, J. C. (2015). A revolução verde e a modernização agrícola na mesorregião noroeste do Rio Grande do Sul–1960/1970. *Revista Teoria e Evidência Econômica*, 21(45), 257-281. https://doi.org/10.5335/rtee.v21i45.6187

Alves, T. A, Pinheiro, P. F., Praça-Fontes, M. M., Andrade-Vieira, L. F, Corrêa, K. B., Alves, T. A., Cruz, F. A, Lacerda Júnior, V., Ferreira, A. & Soares, T. C. B. (2018). Toxicity of thymol, carvacrol and their respective phenoxyacetic acids in *Lactuca sativa* and *Sorghum bicolor*. *Industrial Crops and Products*, 114(1), 59-67. https://doi.org/10.1016/j.indcrop.2018.01.071

Andrade-Vieira, L., Gedraite, L., Campos, J. & Davide, L. (2011). Spent Pot Liner (SPL) induced DNA damage and nuclear alterations in root tip cells of *Allium cepa* as a consequence of programmed cell death. *Ecotoxicology and Environmental Safety*, 74(4), 882-888. https://doi.org/10.1016/j.ecoenv.2010.12.010

Aragão, F. B., Palmieri, M., Ferreira, A., Costa, A., Queiroz, V., Pinheiro, P. & Andrade-Vieira, L. (2015). Phytotoxic and cytotoxic effects of *Eucalyptus* essential oil on lettuce (*Lactuca sativa* L.). *Allelopathy Journal*, 35(2), 259-272.

Aragão, F. B., Queiroz, V. T., Ferreira, A., Costa, A. V., Pinheiro, P. F., Carrijo, T. T. & Andrade-Vieira, L. F. (2017). Phytotoxicity and cytotoxicity of *Lepidaploa rufogrisea* (Asteraceae) extracts in the plant model *Lactuca sativa* (Asteraceae). *Revista de Biología Tropical*, 65(2), 435-443. http://dx.doi.org/10.15517/rbt.v65i2.25696

Bernardes, P. M., Andrade-Vieira, L. F., Aragão, F. B., Ferreira, A., Ferreira, M. F. S. (2015). Toxicity of Difenoconazole and Tebuconazole in *Allium cepa*. *Water Air & Soil Pollution*, 226(1), 207.

Carson, R. (2002). *Silent spring: The classic that launched the environmental movement*. Houghton Mifflin Harcourt.

Carvalho, M. S. S., Andrade-Vieira, L. F., dos Santos, F. E., Correa, F. F., das Graças Cardoso, M. & Vilela, L.R. (2019). Allelopathic potential and phytochemical screening of ethanolic extracts from five species of *Amaranthus* spp. in the plant model *Lactuca sativa*. *Scientia Horticulturae*, 245(1), 90-98. https://doi.org/10.1016/j.scienta.2018.10.001

Cimanga, K., Kambu, K., Tona, L., Apers, S., De Bruyne, T., Hermans, N., Totté, J., Pieters, L. & Vlietinck, A.J. (2002). Correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. *Journal of Ethnopharmacology*, 79(2), 213-220. https://doi.org/10.1016/S0378-8741(01)00384-1

Costa, A. V., Moreira, L. C., Pinto, R. T., Alves, T. A., Schwan, V. V., Queiroz, V. T., Praça-Fontes, M. M., Teixeira, R. R., Moraisa, P. A. & Jesus Júnior, W. C. (2019). Synthesis of Glycerol-Derived 4-Alkyl-Substituted 1, 2, 3-Triazoles and Evaluation of Their Fungicidal, Phytotoxic, and Antiproliferative Activities. *Journal of the Brazilian Chemical Society*, 31(4), 821-832. https://doi.org/10.21577/0103-5053.20190246

Costa, A. V., Oliveira, M. V. L., Pinto, R. T., Moreira, L. C., Gomes, E. M. C., Alves, T. A., Pinheiro, P. F., Queiroz, V. T., Vieira, L. F. A. & Teixeira, R. R. (2017). Synthesis of Novel Glycerol-Derived 1, 2, 3-Triazoles and Evaluation of Their Fungicide, Phytotoxic and Cytotoxic Activities. *Molecules*, 22(10), 1666 (1-15). https://doi.org/10.3390/molecules22101666

Cruz, C.D. (2013). GENES: a software package for analysis in experimental statistics and quantitative genetics. *Acta Scientiarum, Agronomy*, 35(3), 271-276. https://doi.org/10.4025/actasciagron.v35i3.21251

Dhaliwal, H., Thind, T., Mohan, C. (2004). Relative activity of essential oils from plants against *Penicillium digitatum* causing post-harvest fruit rot of Kinnow mandarin. *Plant Disease Research Ludhiana*, 19(2), 140-143.

dos Santos, F. E., Carvalho, M. S. S., Silveira, G. L., Correa, F. F., das Graças Cardoso, M., Andrade-Vieira, L. F. & Vilela, L. R. (2018). Phytotoxicity and cytogenotoxicity of hydroalcoholic extracts from *Solanum muricatum* Ait. and *Solanum betaceum* Cav.(Solanaceae) in the plant model *Lactuca sativa*. *Environmental Science and Pollution Research*, 26(1), 27558-27568. https://doi.org/10.1007/s11356-017-1015-x

Erler, F., Ulug, I. & Yalcinkaya, B. (2006). Repellent activity of five essential oils against *Culex pipiens*. *Fitoterapia*, 77(1), 491-494. https://doi.org/10.1016/j.fitote.2006.05.028

Falahati, M., Omidi Tabrizib, N. & Jahaniani, F. (2005). Anti dermatophyte activities of *Eucalyptus camaldulensis* in comparison with Griseofulvin. *Iranian Journal of Pharmacology and Therapeutics*, 4(1), 80-83.

Fernandes, T. C. C., Mazzeo, D. E. C. & Marin-Morales, M. A. (2009). Origin of nuclear and chromosomal alterations derived from the action of an aneugenic agent—Trifluralin herbicide. *Ecotoxicology and Environmental Safety*, 72(6), 1680-1686. https://doi.org/10.1016/j.ecoenv.2009.03.014

Flores, A. V., Ribeiro, J. N., Neves, A. A. & Queiroz, E. L. R. (2004). Organoclorados: um problema de saúde pública. *Ambiente & Sociedade*. 7(2), 111-124.

Freitas, A. S., Cunha, I. M. F., Andrade-Vieira, L. F. & Techio, V. H. (2016). Effect of SPL (Spent Pot Liner) and its main components on root growth, mitotic activity and phosphorylation of Histone H3 in *Lactuca sativa* L. *Ecotoxicology and Environmental Safety*, 124(1), 426-434. https://doi.org/10.1016/j.ecoenv.2015.11.017

Hendges, C., Schiller, A. P., Manfrin, J., Macedo Jr, E. K., Gonçalves Jr, A. C. & Stangarlin, J.R. (2019). Human intoxication by agrochemicals in the region of South Brazil between 1999 and 2014. *Journal of Environmental Science and Health, Part B*, 54(4), 219-225. https://doi.org/10.1080/03601234.2018.1550300

Kordali, S., Cakir, A., Ozer, H., Cakmakci, R., Kesdek, M. & Mete, E. (2008). Antifungal, phytotoxic and insecticidal properties of essential oil isolated from Turkish Origanum acutidens and its three components, carvacrol, thymol and p-cymene. *Bioresource Technology*, 99(18), 8788-8795. https://doi.org/10.1016/j.biortech.2008.04.048

Leme, D. M. & Marin-Morales, M. A. (2009). *Allium cepa* test in environmental monitoring: a review on its application. *Mutation Research*, 682(1), 71-81. https://doi.org/10.1016/j.mrrev.2009.06.002

Mallard, I., Bourgeois, D. & Fourmentin, S. (2018). A friendly environmental approach for the controlled release of *Eucalyptus* essential oil. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 549(1), 130-137. https://doi.org/10.1016/j.colsurfa.2018.04.010

Melo, B., Pinto, J., Luz, J. M. Q., Peixoto, J. R. & Juliatti, F. C. (2001). Diferentes antioxidantes no controle da oxidação, germinação e desenvolvimento das plântulas na cultura in vitro de embriões da guarirobeira [*Syagrus oleracea* (Mart.) Becc.]. *Ciência e Agrotecnologia*, 25(1), 1301-1306.

Mendes, L. A., Souza, T. S., Menini, L., Guilhen, J. H. S., Bernardes, C. O., Ferreira, A. & Ferreira, M. F. S. (2018). Spring alterations in the chromatographic profile of leaf essential oils of improved guava genotypes in Brazil. *Scientia Horticulturae*, 238(1), 295-302. https://doi.org/10.1016/j.scienta.2018.04.065

Moreira, M., Ponce, A., Del Valle, C. & Roura, S. (2005). Inhibitory parameters of essential oils to reduce a foodborne pathogen. *LWT - Food Science and Technology*, 38(5), 565-570. https://doi.org/10.1016/j.lwt.2004.07.012

Oliveira, J. (1998). *Caracterização da madeira de eucalipto para a construção civil* (1998). 429 p.

Oluma, H. & Garba, I. (2004). Screening of *Eucalyptus globulus* and *Ocimum gratissimum* against *Pythium aphanidermatum*. *Nigerian journal of plant protection*, 21(1), 109-114.

Pereira, J.L. (2010). *Composição química dos óleos essenciais de espécies de Eucalyptus L'Herit (Myrtaceae).* Universidade Federal de Viçosa.

Pinheiro, P. F., Costa, A. V., Alves, T. A., Galter, I. N., Pinheiro, C. A., Pereira, A. F., Oliveira, C. M. R. & Fontes, M. M. P. (2015). Phytotoxicity and Cytotoxicity of Essential Oil from Leaves of *Plectranthus amboinicus*, Carvacrol, and Thymol in Plant Bioassays. *Journal of Agricultural and Food Chemistry*, 63(1), 8981-8990. https://doi.org/10.1021/acs.jafc.5b03049

Ramezani, H., Singh, H., Batish, D. & Kohli, R. (2002). Antifungal activity of the volatile oil of *Eucalyptus citriodora*. *Fitoterapia*, 73(1), 261-262. https://doi.org/10.1016/S0367-326X(02)00065-5

Santos, E., da Silva, V., da Silva, A., Oliveira, B., Oliveira, T. & Azevedo, R. (2019). Perception of risks to the use of agrochemicals by high school students in rural areas in the District of Lucialva, Jauru-MT. *Scientific Electronic Archives*, 12(1), 84-90.

Silva, L., Silva, A., Queiroz, M., Lima, C., Rocha, P. & D'antonino, L. (2012). Action of *Eleusine coracana* in the remediation of soils contaminated with picloram. *Planta Daninha*, 30(3), 627-632. https://doi.org/10.1590/S0100-83582012000300019

Silva, R. C., Neris, M. A., Lopes, M. A., Cerqueira, E. M. M. & Meireles, J. R. C. (2017). Danos cromossômicos e alterações nucleares em células esfoliadas do epitélio gengival de indivíduos com periodontite crônica moderada. *Revista de Ciências Médicas e Biológicas*, 16(1), 19-24. http://dx.doi.org/10.9771/cmbio.v16i1.17728

Silveira, G. L., Lima, M. G. F., Reis, G. B., Palmieri, M. J. & Andrade-Vieria, L. F. (2017). Toxic effects of environmental pollutants: Comparative investigation using *Allium cepa* L. and *Lactuca sativa* L. *Chemosphere*, 178 (1), 359-367. https://doi.org/10.1016/j.chemosphere.2017.03.048

Uhlmann, L. A. C., Oliveira, R. J. & Santos, M. G. (2018). Efeitos alelopáticos de extratos vegetais de *Hancornia speciosa* Gomes na germinação de *Lactuca sativa* L. *Revista Fitos*, 12(2), 147-160. https://doi.org/10.5935/2446-4775.20180014

Vasconcelos, L. C., de Souza Santos, E., de Oliveira Bernardes, C., da Silva Ferreira, M. F, Ferreira, A., Tuler, A. C, Carvalho, J. A. M., Pinheiro, P. F. & Praça-Fontes, M. M. (2019). Phytochemical analysis and effect of the essential oil of *Psidium* L. species on the initial development and mitotic activity of plants. *Environmental Science and Pollution Research*, 26(1), 26216-26228. https://doi.org/10.1007/s11356-019-05912-6.

Xavier, A., Borges, R., Pires, I. E, & Cruz, C. D. (1993). Variabilidade genetica de oleo essencial e de crescimento em progenies de meio-irmaos de Eucalyptus citridora Hook. *Revista Arvore*, 39(6), 1047-1054.