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Evaluation of the cytotoxic and genotoxic potentials of the solubilized extract of the coffee waste using *Allium cepa* as a test system

Avaliação das potencialidades citotóxicas e genotóxicas do extrato solubilizado da borra do café utilizando *Allium cepa* como sistema teste

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Resumo

As biomassas residuais de café e outras bebidas geradas em cafeterias, restaurantes, residências, bares entre outros não possuem registros devido à dificuldade de quantificar esse tipo de resíduo em diferentes locais. Para analisar os efeitos tóxicos da substância ou substâncias misturadas indevidamente descartadas, devem ser realizados testes de toxicidade que visem prever o impacto potencial de um xenobiótico no meio ambiente. O objetivo deste trabalho foi avaliar o impacto do extrato solubilizado de resíduos de café na germinação e desenvolvimento de mudas de cebola (A. cepa), bem como avaliar o potencial citotóxico e genotóxico deste extrato em células meristemáticas de raiz de cebola. O delineamento experimental foi inteiramente casualizado, composto por cinco tratamentos para o grupo teste e dois controles. Para todos os tratamentos, 5 repetições foram utilizadas. Para avaliar o teste de citotoxicidade e genotoxicidade, 5 plântulas de cada tratamento foram retiradas após o processo de germinação. Assim, a partir do tratamento T2, o extrato foi capaz de proporcionar um efeito deletério na germinação e crescimento radicular. O extrato apresentou também efeito genotóxico na concentração dos tratamentos T2 a T5 e, além da genotoxicidade, os tratamentos T3 a T5 também apresentaram efeito citotóxico.

***Palavras-chave**: Citotoxicidade; Genotoxicidade; Borra de café*

Abstract

The residual biomasses of coffee and other beverages generated in coffee shops, restaurants, residences, bars among others do not have registers due to the difficulty of quantifying this type of waste in different places. To analyze the toxic effects for substance or mixed substances improperly discarded should be performed toxicity tests that aim to predict the potential impact of a xenobiotic on the environment. The objective of this work was to evaluate the impact of the solubilized extract of coffe waste on the germination and development of onion seedlings (A. cepa), as well as to evaluate the cytotoxic and genotoxic potential of this extract in onion root meristematic cells. The experimental design was completely randomized, consisting of five treatments for the test group and two control group. For all treatments, 5 replicates were used. To evaluate the cytotoxicity and genotoxicity test, 5 seedlings of each treatment were removed after the germination process. Thus, from the treatment T2, the extract was able to provide a deleterious effect on germination and root growth. The extract presented a genotoxic effect in the concentration of T2 to T5 treatments, and in addition to genotoxicity, T3 to T5 treatments also showed a cytotoxic effect.

***Keywords**: Cytotoxicity; Genotoxicity; Coffee waste*

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1 Introduction

The residual biomasses of coffee and other beverages generated in coffee shops, restaurants, residences, bars among others do not have registers due to the difficulty of quantifying this type of waste in different places (FREITAS, 2000; VEGRO e CARVALHO, 2006; RAMALAKSHMI et al., 2009; PANUSA et al., 2013).

The chemical and physical characterization of these elements points out its contaminating potential, however, it is fundamental to evaluate its biological implications and possible interactions. While the chemical analyzes identify and quantify the concentrations of toxic substances, toxicity tests assess the effect of these substances on biological systems (COSTA et al., 2008; KALCIKOVA et al., 2011).

In addition, according to Technical Standard NBR 10.004 Solid waste – Classification(ABNT, 2004a), this waste is classified as: Class II A, Not Hazardous - Not Inert, however soluble in water.

The main objectives of the ecotoxicology are to identify the risks associated with a substance and to determine under what conditions of exposure these risks are induced. About eleven million chemicals are known and only a small percentage is well studied in relation to their effects and dynamics in the environment (ZAKRZEWSKI, 1994; MANNING and TIENDMANN, 1995; HODGSON, 2004).

To analyze the toxic effects for substance or mixed substances improperly discarded should be performed toxicity tests that aim to predict the potential impact of a xenobiotic (toxic agent) on the environment (FLOHR et al., 2005).

In this context, germination and seedling root development trials have been used to evaluate and quantify the toxicity of water-soluble compounds as well as mixtures of complex substances, leachate, sediments among others (BOWERS, et al., 1997). These substances are submitted to acute phytotoxicity tests, aiming at evaluating both lethal effects through inhibition of seed germination and sublethal effects via root development (USEPA, 1996; OECD, 2003). The objective of this work was to evaluate the impact of the solubilized extract of coffe waste on the germination and development of onion seedlings (*Allium cepa*), as well as to evaluate the cytotoxic and genotoxic potential of this extract in onion root meristematic cells.

Allium cepa is a test system validated by the United Nations Environment Program (UNEP) and the International Program on Chemical Safety (IPCS, WHO) for the *in-situ* monitoring of cytotoxicity and genotoxicity of environmental substances (Bagatini et al., 2007). According to Fiskejo (1985) positive results in *A. cepa* should be considered with an indication that the sample tested may also be a biological hazard to other organisms by detecting the presence of cytotoxic and/or genotoxic substances in the environment. El-Shahaby et al. (2003) reinforce the importance of using the test in *A. cepa* for the detection of toxicity/genotoxicity and the importance as an indicator for human health. In this way, several authors in environmental monitoring have used this methodology(Rank and Nielsen, 1993; Nielsen and Rank, 1994; Grover and Kaur, 1999; Leme and Marin-Morales, 2008; Hoshina and Marin-Morales, 2009; Radic et al., 2010; Oliveira et al., 2011; Gomes et al., 2015; de Castro and Sousa et al., 2017; Kasper et al., 2018).

The objective of this work was to evaluate the impact of the solubilized extract of coffe waste on the germination and development of onion seedlings (*A. cepa*), as well as to evaluate the cytotoxic and genotoxic potential of this extract in onion root meristematic cells.

2 Materials and methods

2.1. Preparation and production of the extract

Samples of the coffe waste were collected according to the methodology recommended by NBR 10.006 (ABNT, 2004b). Due to the homogeneity of the physical appearance of the waste material, four single top, middle and base samples were collected around the waste pile. These were mixed in order to obtain a composite sample. The solid material of the composite sample was fractionated into pieces to obtain uniform fragments, giving rise to a homogeneous sample. From this, the process of obtaining the solubilized waste coffe extract was started.

The solubilized extract was prepared according to the methodology recommended by NBR 10.006 (ABNT, 2004b). To prepare the solubilized extract, 250 g of waste coffe were transferred to a hermetically sealed glass container containing 1.0 L of distilled water. The material (water + waste coffe) was stirred for 5 min on a magnetic stirrer (Fisatom 752) at room temperature (25° C ± 1). Thereafter, the vessel was closed and the material was allowed to stand for 7 days. After this period the filtration occurred through a filter membrane of 0.45 μm.

2.2. Sample of seeds

Seeds of onion BaiaPeriform*A. cepa* of the ISLA PAK brand were obtained with 99.7% purity, 98% germination and validity until August / 19. They were used directly, without performing any previous disinfection procedure or break of dormancy.

2.3. Phytotoxicity test

Toxicity tests on germination and root growth using onion seeds (*Allium cepa*) were carried out to evaluate the lethal effects, from the inhibition on seed germination and the sublethal effects through root development (SOBRERO; RONCO, 2004; OECD, 2003).

The experimental design was completely randomized, consisting of five treatments for the test group and two control group. For all treatments, 5 replicates were used. The treatments proposed in the test group were: T1 = 3.125% solubilized + 96.875% deionized water; T2 = 6.25% solubilized + 93.75% deionized water; T3 = 12.5% solubilized + 87.5% deionized water; T4 = 25% solubilized + 75% deionized water; T5 = 50% solubilized + 50% deionized water. The TC (control group) consisted of only 4.0 mL of distilled water, plus the TP (positive control) with ethylmethanesulfonate (EMS, 25 mM).

Additionally, 10 seeds of onion were placed in a Petri dish of 9.5 cm in diameter and moistened with the solution prepared according to established treatments, having as substrate a qualitative filter paper (porosity 14 µm). The Petri dishes were conditioned in a BOD incubator incubator with photoperiod (CIENLAB and model CE-300/350-F) at a temperature of 25 ± 1 ºC for 12 h light / 12 h dark.

2.4. Phytomass evaluation

At the end of 120 h (5 days), the following parameters were evaluated: number of germinated seeds and length of rootlets (USEPA, 1996; OECD, 2003).

In order to determine the sublethal effects, it was sought to identify the concentration of unobserved effect (NOEC), which corresponds to the highest concentration / concentration of toxic agent that does not cause deleterious effect statistically significant in the organisms in the time and in the conditions of the test; and the observed effect concentration (LOEC), which corresponds to the lowest concentration / concentration of toxic agent that causes statistically significant deleterious effects on organisms at the time of exposure and the test conditions (RONCO, BAEZ and GRANADOS, 2004). The determination of NOEC and LOEC were carried out from the comparison between the means of the root length.

2.5. Citotoxicity and genotoxicity test

To evaluate the cytotoxicity and genotoxicity test, 5 seedlings of each treatment were removed after the germination process. In addition, the root apices presenting between 1.5 and 2.5 mm were removed. After removal, the apices were washed in distilled water, fixed in 3:1 (V/V) ethanol: glacial acetic acid solution and stored at 4 °C, where they remained until the time of preparation of the slides. The slides were made according to Iganciet al (2006) with modifications. For the preparation of the slides the tips were washed with distilled water in two 5 minutes cycles, hydrolyzed in 5N HCl for 30 minutes and again washed with distilled water in two 5 minutes cycles. The root apices were fragmented with the aid of a razor slide, stained with 2% acetic orcein and the cover slip was placed and pressed onto the material. Then the slides were sealed with enamel.

2.5.1 Slide analysis

The slides were blindly viewed under a light microscope at 400X magnification. The presence of chromosomal alterations and aberrations was analyzed in 1000 cells per treatment. At the end, the mitotic index (MI) was calculated (Pires et al., 2001).

2.6. Statistical analysis

The germination tests measurements and the length of rootlets were analyzed using a graduated ruler and a digital caliper. After these measurements, the parameters averages were calculated and Tukey's test used to evaluate the significance at 0.05 (5% probability) among them (COSTA NETO, 1977; MILLER; MILLER, 1993).

Statistical analysis of the data obtained in the cytotoxicity and genotixicity test using the *Alliumcepa* test was performed using the qui-quadradotest (GOMES, 1982).

In order to determine the existence of a correlation between seed germination, lenght of rootles and the introduction of the solubilized coffee waste extract, was used the Pearson correlation coefficient, with determination of the correlation index (p), in order to to verify if there is correlation with statistical significance (ACHEN, 1977; ALDRICH, 1995; ZAR, 1996).

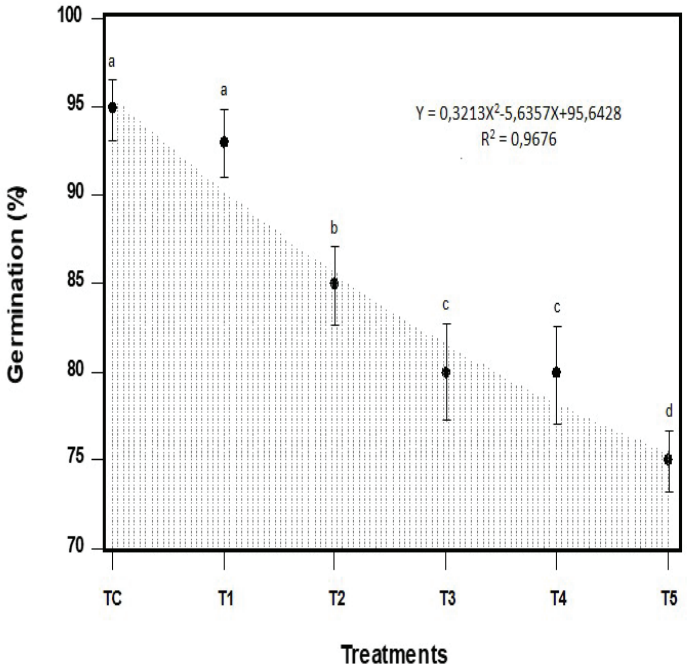
All calculations and graphs presented in this work were performed by BioEstat 5.0 and SigmaPlot 12.5 software.

3 Results and discussion

The average values of the germination percentage as a function of the different treatments with the solubilized extract are shown in Figure 1. The control group presented 95% germination. By the other hand, the treatment T2 presented 85% and T5

75% germination. It was verified that from the concentration administered in T2 there was an inhibition in the germination of 12% compared the control group. The same inhibitory behavior was observed for the other treatments (T3 and T4, 19%; T5, 27%). Significant differences were observed between the control group and test group for theTukey test ($p<0.05$). The quadraticregression describes a decreasing behavior for the germination response in relation to the different treatments with the solubilized extract.

Figure 1 - Evaluation of the average germination percentage of seeds of onion (Allium cepa) contaminated with different concentration of the solubilized extract from the coffee waste.



TC = deionized water control; T1 = solubilized concentration of 3.125%; T2 = solubilized concentration of 6.25%; T3 = solubilized concentration 12.5%; T4 = solubilized concentration of 25%; T5 = solubilized concentration of 50%. Means followed by the same lowercase letter do not differ statistically from each other by Tukey test (p◎0,05).

Shafaei et al. (2014) reports that the germination process depends on the presence of water to the seed matrix, the oxygen content and some external environmental factors such as light and temperaturas. Thus, these results indicate that the different concentrationof coffe waste exerted influence on the percentage of germination of onion seeds (*Allium cepa*).

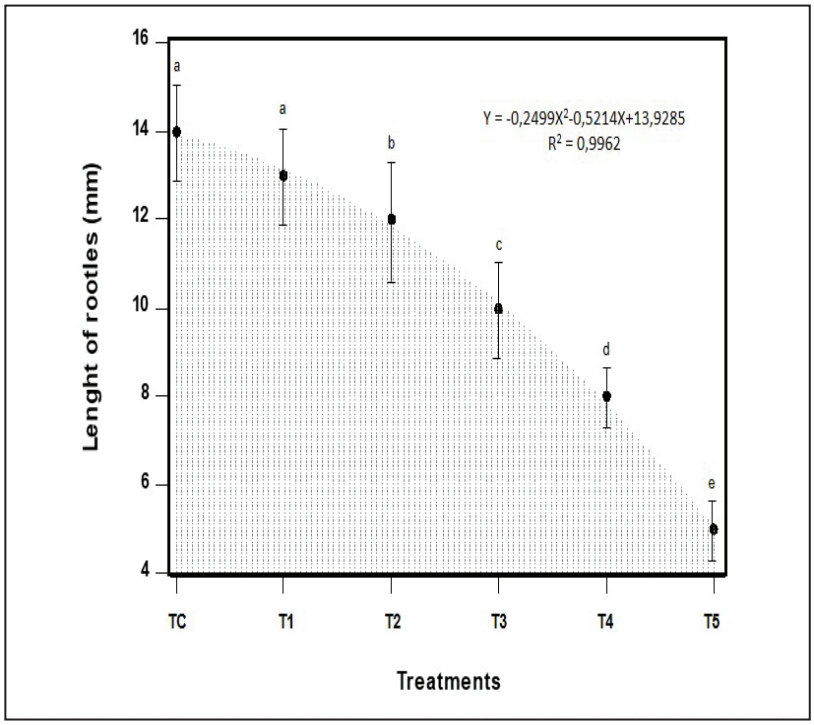
The medium values of root structure growth in function of the different treatments with the solubilized extract are shown in figure 2. The control group showed a growth of 14 mm. As in germination, It was verified that from the concentration administered in T2 there was an inhibition in the germination of 14% compared the control group. The same inhibitory behavior was observed for the other treatments (T3, 28%; T4, 42%; T5, 64%). Significant differences were observed between the control group and test group for theTukey test ($p<0.05$). The quadratic regression describes a decreasing behavior for the lenght of rootles growth response as a function of the different treatments with the solubilized extract.

It is observed that as the concentration were increased, presented a more significant effect on the radicle growth when compared to the germination parameter. The evaluation of the effect on lenght of rootles may reflect the toxicity of the soluble compounds present at such low concentration levels that they are not sufficient to inhibit germination but may delay or inhibit the lenght of rootles process depending on the mode and site of action of the compounds. Thus, inhibition of lenght of rootles shows a sensitive sublethal indicator for evaluating biological effects on plants (SOBRERO e RONCO, 2004; TAMADA et al., 2012). Thus, in the present study, the same effect produced on onion seeds (*Allium cepa*) was observed, mainly because the pollutant is high solubility in water.

The NOEC for the germination was identificated in the treatment T1, because this was the concentration that did not present significant difference of the control group. However, the determination of LOEC was established in the treatment T2, because this was the lowest concentration able to promote subtly perceptible effects by inhibiting the of the germination. The same behavior was observed for the length of rootles. The NOEC was established in the treatment T1 and LOEC in T2.

There was a highly significant negative correlation for the physical variables under study. For the inhibition of germination, the value (r) found was -0.973. In the same way, for inhibition of lenght of rootles the value (r) found was (r) -0.981. That is,

Figure 2 - Radicle lenght average of rootles on the germination of seeds of onion (*Allium cepa*) contaminated with different dosages of the solubilized extract from the coffee waste.



TC = deionized water control; T1 = solubilized concentration of 3.125%; T2 = solubilized concentration of 6.25%; T3 = solubilized concentration 12.5%; T4 = solubilized concentration of 25%; T5 = solubilized concentration of 50%. Means followed by the same lowercase letter do not differ statistically from each other by Tukey test (p⊚0,05).

98% associated with the introduction of the pollutant and 2% the random variables to the system. The results confirms the existence of a strong negative correlation.

Thode-Filho et al (2017) evaluated of the impact of the solubilized extract of coffe waste on the germination of cabbage seeds (*Brassica oleracea var. capitata*). There was a negative effect on the germination rate and root growth for extract, of the concentration s tested. It was verified that from the lowest dosage administered (0.1 mL) there was an inhibition in the germination of 30%. The determination of LOEC was established in the treatment with 0.1 mL of the extract. It is verified that for the present study NOEC was established in T1 treatment and the LOEC was established in T2 (0.25 mL). However, verified that the seeds onin were a little more tolerant of the pollutant than the cabbage.

In relation to the cytotoxic and genotoxic analysis, presented in table 1, the results showed that, except for the T1 treatment, the solubilized extract of coffee waste in the concentration of T2 to T5 treatments, presents genotoxic and cytotoxic potential.

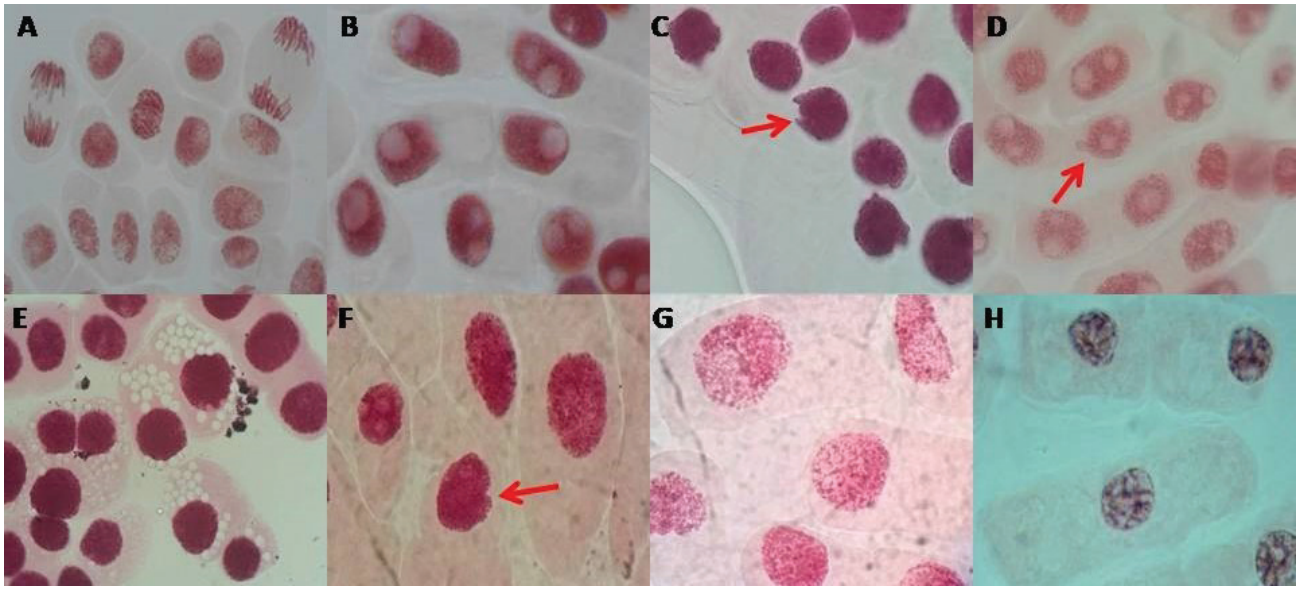
Table 1. Cell changes and mitotic index in *A. cepa*submitted to different treatments.

TRAT.	BN	NG	NE	NOT	CX	AN	MI%
TC	122^{e}	264^{b}	14^{b}	12^{c}	192^{c}	0^{b}	$11,2^{c}$
TP	199^{b}	50^{e}	2^{d}	10^{c}	403^{a}	58^{a}	0^{e}
T1	106^{f}	148^{d}	11^{b}	31^{b}	74^{e}	0^{b}	$10,9^{c}$
T2	$207^{a,b}$	194^{c}	0^{e}	0^{e}	140^{d}	0^{b}	$12,4^{b,c}$
T3	213^{a}	206^{c}	43^{a}	50^{a}	0^{g}	0^{b}	16^{a}
T4	157^{d}	254^{b}	4^{c}	5^{d}	346^{b}	0^{b}	$13,4^{b}$
T5	185^{c}	385^{a}	0^{e}	0^{e}	35^{f}	0^{b}	$5,9^{d}$

(a) – (g) different letters in the same column differ from each other (P<0,05) according to the χ^2 test; TC = deionized water control; TP = positive control; ethyl methanesulfonate (25 mM); T1 = treatment concentration of 3.125%; T2 = treatment concentration of 6.25%; T3 = treatment concentration of 12.5%; T4 = treatment concentration of 25%; T5 = treatment concentration of 50%. NB – nuclear bud; NC – Nucleoli changes; NE – Necrosis; NOT – Notched nucleus; CL – karyorrhexis-like; NA – nuclear abnormalities; MI – mitotic index. 1000 cells for each treatment were analyzed.

In T2, T3, T4 and T5 treatments a significantly larger number of cells with nuclear buds were observed when compared to the negative control (Figure 3C and 3A). In addition, the T5 treatment also showed a significantly higher number of cells whit nucleolar changes compared to the negative and positive controls (Figure 3B and 3D), whereas the T3 treatment showed a significant number of cells with notched nucleus (Figure 3F).

Figure 3 - Changes in *A. cepa* cells submitted to different treatments: A – normal cells (negative control); B – nucleoli changes (positive control); C – nuclear bud (arrow); D – nucleoli changes and nuclear bud (arrow); E – necrosis; F – notched nucleus (arrow); G – karyorrhexis-like; H – nuclear abnormalities



These alterations are characteristic of cells that have been exposed to genotoxic agents, such as the positive control used in this work (MONTANARO et al., 2008; FENECH et al., 2011; KUMAR et al., 2015).

The presence of nuclear buds, as well as other nuclear abnormalities, is indicative of genotoxic effect and chromosomal instability. It has been observed in cell cultures submitted to conditions that induce gene amplification (FENECH et al., 2011). Shimizu et al. (1998, 2000) working with mammalian cells demonstrated that the amplified DNA is selectively located at specific sites in the periphery of the nucleus being eliminated through nuclear buds formation during the S phase of the cell cycle.

In addition, the morphometric and quantitative analysis of the nucleoli has been used as an auxiliary tool to distinguish between benign and malignant cells in several animal tissues (MAKINEN et al., 1993; KRUGER et al., 2000). In these cases, irregular and hypertrophied nucleoli are indicative of malignancy (MONTANARO et al., 2008). Similar studies have been carried out in plant cells, relating the variation in size and shape of nucleoli with the action of genotoxic agents (ARKHIPCHUK et al., 2000; VENTURA-CAMARGO et al., 2011).

The occurrence of notched nuclei has also been used as an indication of the presence of components with genotoxic potencial (PAKRASHI et al., 2014; KANNANGARA and PATHIRATNE et al., 2015; BRAHAM et al., 2017; VIANA et al., 2018). Although the pathways of induction and formation have not yet been fully elucidated, the formation of notched nuclei can be attributed to changes in cytoskeletal proteins responsible for the maintenance of the nuclear form (GHISI et al 2014).

In addition to genotoxicity, T3, T4 and T5 treatments also showed changes that indicate the cytotoxic potential of the extract. The T3 treatment presented a significant number of cells in necrosis, whereas the T4 treatment presented a high number of cells with characteristics similar to karyorrhexis, these being called karyorrhexis-like.

Necrosis is a phenomenon that can result from the action of toxic components which leads to metabolic damage, membrane ruptures and consequent cell lysis (ZAKERI AND LOCKSHIN, 2002; HOSHINA AND MARIN-MORALES, 2009), whereas karyorrehexis is characterized by an irregular distribution of the chromatin, being found in cells that are in death process by necrosis or apoptosis (THOMAS et al., 2007; THOMAS et al., 2009; SULCZEWSKI et al., 2014).

Mitotic index is also used as an indicator of cytotoxicity, which is determined by the increase or decrease of mitotic index (VENTURA-CAMARGO et al., 2011; SOUZA et al., 2018).

Treatments that showed significant differences with respect to the negative control were T3, with a larger number of cells in division, and T5 which presented a substantial reduction of the mitotic index. The other treatments did not present signifi-

cant differences in relation to the negative control. Adverse effects on the mitotic index, similar to those observed with the T3 and T5 treatments, have already been found by other authors, and can be explained by the existence of variable interactions between the components of an aqueous extract at different concentrations, which can lead to a different physiological action according to the dose applied (BORGES et al., 2007; BORELLA et al., 2011; TEIXEIRA et al., 2017) .

It is important to stress that although T3 treatment had a higher mitotic index than the negative control, there was also a higher number of cells in necrosis, which can in part explain the reduction in root growth. Similarly, the larger number of cells karyorrhexis-like in T4 treatment can also contribute to explain the reduction observed in the root growth analysis.

4 Conclusion

The coffee waste are classified as non-hazardous and non-inert due to their solubility. Therefore, its final destination must employ special measures of collection and protection to the environment.

There was a negative effect on the germination and root growth rate of the extract under study, due to the increase in the concentrations tested.

NOEC for germination was identified in T1 treatment and LOEC was established in T2 treatment. The same behavior was observed for the lenght of rootles. NOEC was established in T1 and LOEC treatment in T2.

Thus, from the concentration of 6.25% (T2 treament), the solubilized extract was able to provide a deleterious effect on germination and root growth. In the same way, the extract presented a genotoxic effect in the concentration of T2 to T5 treatments, and in addition to genotoxicity, T3, T4 and T5 treatments also showed a cytotoxic effect.

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