Fronteiras: Journal of Social, Technological and Environmental Science
http://periodicos.unievangelica.edu.br/fronteiras/
ISSN 2238-8869

Article

The Toxicological Profile of the Agrotoxic Acid 2,4 Dichlophenoxyacetic in Fleischmann® Yeast

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ABSTRACT

Pesticides are chemical substances used to ensure crop productivity. However, they can cause numerous consequences for the environment. As the 2,4-D, which even when applied with control can cause great environmental changes, because when dispersed in the environment, it reaches the different compartments of the ecosystem. Organisms absorb these substances and with bioaccumulation, deleterious effects occur that are difficult to detect, requiring test organisms that offer rapid responses. Thus, this study aims to evaluate the cytotoxicity and genotoxicity effect of the 2,4 D pesticide on the yeast Fleischmann® and verify its application as a possible bioindicator organism. Cytotoxicity assays were performed through cell growth, using 2,4-D at concentrations (2.0, 4.0 and 6.0 μg L^{-1}) in which the yeast FLE was incubated and at times (30, 60 and 90 min) 5μL aliquots were dropped into 96-well plates and incubated at 30°C for 72 hours. Genotoxicity was evaluated by the comet assay method and the results were presented according to the levels of DNA damage. The toxic action of the 2,4-D compound was verified at all concentrations analyzed, and within 90 min there was a greater inhibition of cell growth. The comet assay showed a greater amount of DNA damage in the yeast FLE at the concentration of 6 μg L^{-1}. Yeast FLE showed potential to be used as a bioindicator of chemical compounds in environmental analysis.

Keywords: *Saccharomyces cerevisiae*; comet assay; bioindicator; toxicity; genotoxicity.

RESUMO

Os agrotóxicos são substâncias químicas utilizadas para garantir a produtividade das culturas. Contudo, podem causar inúmeras consequências para o meio ambiente. Como o 2,4-D, que mesmo sendo aplicado com controle pode causar mudanças ambientais, pois quando disperso no ambiente atinge os diferentes compartimentos do ecossistema. Os organismos absorvem essas substâncias e com a bioacumulação ocorrem efeitos deletérios que são difíceis de detectar necessitando de organismos testes que ofereçam respostas rápidas. Assim, este estudo visa avaliar o efeito de citotoxicidade e genotoxicidade do agrotóxico 2,4 D na levedura Fleischmann® e verificar sua aplicação como um possível organismo bioindicador. Os ensaios de citotoxicidade foram através do crescimento celular, sendo utilizado o 2,4-D nas concentrações (2,0, 4,0 e 6,0 μg L^{-1}) nas quais foram incubadas a levedura FLE e em tempos (30, 60 e 90 min) alíquotas de 5μL foram gotejadas em placas de 96 poços e incubadas a 30 °C por 72 horas. A genotoxicidade foi avaliada pelo método ensaio do

Submissão: 23/09/2021 Aceite: 16/05/2022 Publicação: 02/08/2022

v.11, n.2, 141-149. 2022 • p. 141-149. • DOI http://dx.doi.org/10.21664/2238-8869.2022v11i2.p141-149.

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cometa sendo os resultados apresentados conforme os níveis de danos ao DNA. A ação tóxica do composto 2,4-D foi verificada em todas as concentrações analisadas sendo que no tempo de 90 min houve uma maior inibição do crescimento celular. O ensaio do cometa mostrou maior quantidade de danos no DNA da levedura FLE na concentração de 6 μg L^{-1}. A levedura FLE apresentou potencial para ser empregada como um organismo bioindicador de compostos quimicos em análises ambientais.
Palavras-chave: *Saccharomyces cerevisiae*; ensaio do cometa; bioindicador; toxicidade; genotoxicidade.

1. Introduction

Pesticides are chemical substances developed to ensure the productivity of crops from planting to harvest, but their continued use has been the cause of numerous consequences for the environment and still affects non-target organisms (Nsibande & Forbes 2016). However, the most reported polluting actions come from domestic waste, petrochemicals, among others that, when disposed of incorrectly, cause various harm to health and the environment, as they can be found in various environmental compartments such as water, air and soil (Sisinno & Oliveira-Filho 2013).

According to Sarabia et al. (2019), some of the active ingredients consumed in Brazil are glyphosate in the first place, which is classified as low toxic, followed by 2,4-D (2,4-dichlorophenoxyacetic acid), an extremely toxic ingredient. Both are herbicides widely used in crops such as soybean, corn, cotton, rice, sugarcane, coffee, wheat, in pastures and others, being used in the pre-planting and desiccation operation of weeds, which may result in increase productivity of crops and reduction of costs to the producer, however, can cause changes in the environment (De Souza et al. 2020).

Although 2,4-D is an efficient and widely used herbicide, possibly its indiscriminate use can induce the appearance of weeds and organisms resistant to the compost, leading to numerous toxicological problems, as it affects not only target organisms (Pileggi et al. 2020; Singh et al. 2020). Thus, even when applied with control, it can cause substantial ecological changes, considering that there is a dispersion in the environment, reaching some organisms that absorb these substances, causing bioaccumulation that results in deleterious effects, which are difficult to detect, as they tend to manifest themselves as a result of prolonged exposure (Lushchak et al. 2018).

Herbicides, according to De Souza et al. (2020), are synthetic chemical substances that are applied in the interest of promoting the control and death of unwanted pests, such as weeds. Thus, in target organisms they act directly on physiological activities causing loss of viability (Jayaraj et al. 2016), as they act by blocking the biosynthesis of amino acids, interrupting the photosynthesis process in weeds, whereas in non-target organisms they cause persistent toxic effects as they act on cell nutrient transporters, as they interfere with the entry of nutrients into the cell (Pileggi et al. 2020).

The 2,4 D is a weak acid, it is highly lipophilic and, in some organisms, it easily crosses the plasma membrane by passive diffusion, triggering several disturbances, including acidification of the cytosol and oxidizing action (Takano et al. 2019). In yeasts, they can act in several response pathways such as CAMP, TOR, ROS, PKA, triggering defense mechanisms such as gene induction, inhibition of cell growth and loss of viability. In yeasts, the transport of nutrients and other compounds are complex, involving different membrane transporters and depending on the chemical nature of the molecule, it occurs passively or actively and alters numerous cell pathways with important functions for maintaining cell integrity (Claus et al. 2019).

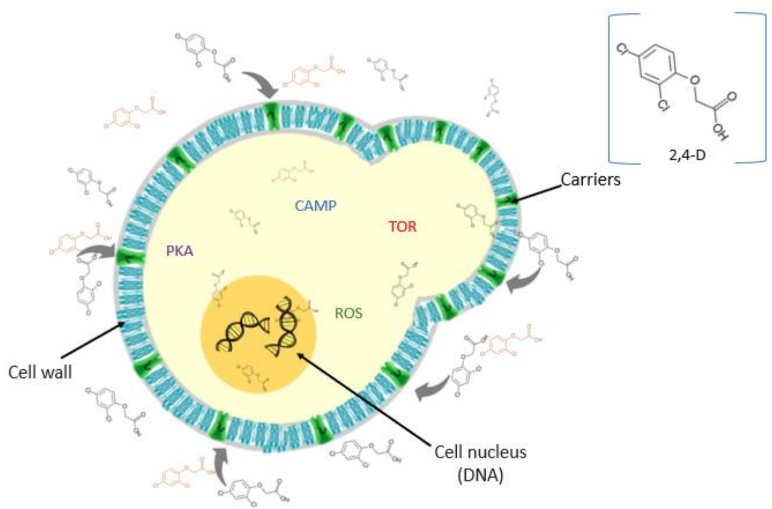

Figure 1. Ion and molecule transport scheme and yeasts. Source: Prepared by the authors.

Among the microorganisms, *Saccharomyces cerevisiae* has been shown to be susceptible to the action of toxic agents, showing changes in its physiological and genetic mechanisms, such as the derepression of genes that trigger numerous defense mechanisms, so in the presence of xenobiotics, there may be the induction of several mechanisms aimed at yeast survival (Moreno-García et al. 2018). The response profile to changes at the gene level in eukaryotes has been considered an important expressive tool in the identification of biomarkers related to the toxic action of these compounds. In fact, *S. cerevisiae* has been used in different areas of knowledge to conduct tests in studies aimed at chemical substances, as it presents a fast and efficient response in the presence of such compounds (Gil et al. 2014).

The evaluation of gene expression in yeast is efficient, as it indicates molecular and physiological changes with different attributions to stresses. *S. cerevisiae* has been used since antiquity and is now considered a pioneering microorganism due to its versatility and application potential, mainly in research focused on genomics, post-genomics and more recent in bioinformatics (North & Vulpe 2010). Such methodological applications have effectively and innovatively contributed to knowledge about gene expression and protein production (Smith et al. 2010; Botstein & Fink 2011).

Transformations in the gene, protein and metabolic profile occur when yeast is exposed to xenobiotic compounds. Such knowledge has provided relevant information regarding the changes that occur in cellular mechanisms against numerous toxic agents (North & Vulpe 2010). In this context, *S. cerevisiae* provides an opportunity to demonstrate the action of numerous toxic compounds, as it encompasses in its genome several response mechanisms. However, a number of genes and pathways are activated in the presence of an agent of this nature. In yeasts, membrane transporters are responsible for detecting and signalling the presence of chemical compounds present in the medium and transporting them to the intracellular environment (Dos Santos & Sá-Correia 2015).

However, studies aimed at chemical compounds that may compromise natural resources causing biotic and abiotic changes in ecosystems in relation to the most different trophic levels and impacting the harmonious relationship of the environment is necessary. Thus, the search for tools that can be used directly and efficiently and that allow the application in different environmental matrices and have a quick response is of interest to researchers working in this area. From this perspective, this study aims to evaluate the cytotoxicity and genotoxicity effect of the 2,4 D pesticide on the yeast Fleischmann® and verify its application as a possible bioindicator organism.

2. Material and methods

2.1. Location of study development

The study as well as the tests were carried out at the Laboratory of Biotechnology, Biochemistry and Biotransformation of the Centro de Estudos em Recursos Naturais - CERNA of the Universidade Estadual de Mato Grosso do Sul - UEMS/Dourados-MS.

2.2. Microorganism used

For this study, the commercial yeast strain *Saccharomyces cerevisiae* Fleischmann®, which is found in the Laboratory of Biochemistry, Biotechnology and Biotransformations of CERNA, was used.

2.3. Preparation of pesticide solutions

The pesticide 2,4-dichlorophenoxyacetic acid (2,4-D) of an analytical grade of 99.9% PA was used. A stock solution was prepared using 100 mg L^{-1} that were diluted in the proportion of 1 mL to 99 mL of methanol to obtain a final concentration of 1000 μg L^{-1} for a final volume of 100 mL. The solution was kept in an amber bottle and stored at low temperature (-18ºC).

2.4. Cytotoxicity and genotoxicity assays

2.4.1. Sample preparation

Yeast was grown in the presence of a 2,4-D pesticide at concentrations (2.0, 4.0 and 6.0 μg L^{-1}), at a temperature of 30 ºC at times of 30, 60 and 90 minutes. In addition to the genotoxicity test, a positive control (C+) with hydrogen peroxide (3%) was performed and negative control (C-) free from stressing compounds.

2.4.2. Cell Growth

After the incubation period, 5 μl aliquots of the samples were dropped into sterile 96-well polyethylene plates containing 2% YPD solid medium containing 1.0% yeast extract (p v^{-1}); 1.0% peptone (p v^{-1}) and 2.0% glucose (p v^{-1}) being incubated at 30 ºC for 72 hours. The samples were analyzed through the colony growth profile through visual observation, being a qualitative method.

2.4.3. Comet Test

The comet assay was standardized based on modifications from the studies by Lah et al. (2004) and Da Silva (2007) and consisted, initially, in washing the slides with Extron detergent (2%), dried at room temperature for 24 hours. After this period, the slides were coated using normal melting point (NMP) agarose at 1%. Then they remained in a suitable place for overnight drying and a second layer was placed using NMP agarose at 0.5%, and solidified in ice. The 5.0 μl samples of cells previously resuspended in S-buffer (1M Sorbitol and 25mM KH_2PO_4) were added and mixed in an Eppendorf containing 70 μl of 0.5% low melting point agarose (LMP), 2 mg mL^{-1} of the Lyticase enzyme (Sigma-Aldrich). Then, the reaction mixture was placed on previously coated slides, covered with coverslips and incubated at 30 ºC for 2 hours to degrade the cell wall through the action of the enzyme. After this period, the slides were placed at a low temperature (4 ºC) for enzyme inactivation. The coverslips were then removed and the slides immersed in 0.5% NMP agarose. Subsequently, the slides were placed in ice-cold lysis solution (30mM NaOH, 1M NaCl, 0.1% N-lauroylsarcosine, 100mM DMSO, 1% Triton-X100) for 1 hour in the absence of light. Then, they were washed in a solution containing 30mM NaOH and 2mM EDTA at pH 13 and immersed in the same buffer in the electrophoresis vat for 20 min to allow DNA denaturation.

The electrophoretic run was performed at 40 min, 25 V and 300 mA under a frosted surface in the absence of light. Then, the slides were neutralized in 400 mM Tris-HCl buffer at pH 7.5 for 15 min, then washed and dried at room temperature and fixed in a fixative solution (15% acetic acid, 5% zinc sulfate and 5% glycerol), were stained in a staining solution (5% calcium carbonate, 0.1% ammonium nitrate, 0.1% silver nitrate, 0.25% tungstosilicic acid and 0.15% formaldehyde) and promptly, washed with distilled water and submerged for 5 min in stopping solution (1% acetic acid). One hundred randomly selected nucleoids were evaluated by optical microscopy and analyzed within five classes of DNA damage (0, 1, 2, 3 and 4) according to the intensity and drag pattern of the degraded genetic material, in which 0 refers to the lowest level of degradation and 4 at the highest level (Figure 2).

2.4.3. Analysis of results

The results were analyzed with Excel 2016 software and the graphs were plotted on GraphPadPrism 7. The DNA damage index (DDI) was analyzed based on the methodology of Fernandes et al. (2015). The experiments were carried out in triplicates.

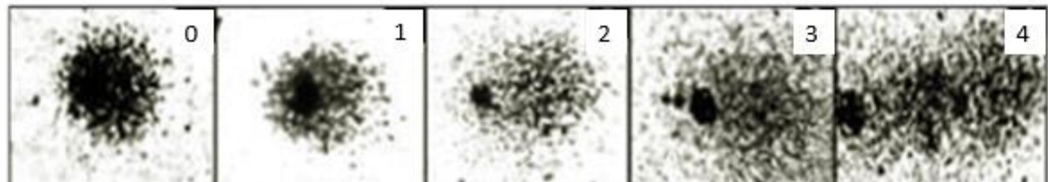

Figure 2. Sequence of image analysis of deoxyribonucleic acid-DNA damage levels classified as (0.1, 2, 3, 4) of the Fleischmann® yeast. Source: Author.

3. Results and discussion

In evaluating the cytotoxic action of the 2,4-D pesticide against the yeast Fleischmann® (FLE), it was observed that the compound had a toxic action and that it distinctly affected the growth profile. Within 30 minutes, the yeast was resistant to the action of the compound and showed growth at all concentrations analyzed, however within 60 minutes the yeast FLE showed moderate sensitivity to the action of 2,4-D with altered growth. Within 90 minutes, the yeast showed severe growth inhibition (Table 1). Probably in the longer time of exposure to 2,4-D, there may have been a greater interaction at the level of cellular structures and pathways, this fact suggests that the compound proved to be toxic to yeast.

Table 1. Evaluation of the cytotoxic action of 2,4-D on the cell growth of the yeast Fleischmann®.

	Concentrações (µg L^{-1})			
Tempo (min)	Controle	2,0	4,0	6,0
30	+	+	+	+
60	+	+++	+++	+++
90	+	++++	++++	++++

Source: Author. (+) growth (++) mild inhibition (+++) moderate inhibition (++++) severe inhibition.

Studies performed using bioassays with *S. cerevisiae* exposed to pesticides, including diuron, dicamba, mecoprop, atrazine, terbutrin, acetamiprid, 2,4-D, showed a cytotoxic effect on yeast (Westlund & Yargeau, 2017). Furthermore, transcriptional analysis studies revealed that the use of xenobiotic compounds act as a stressor and play a repressive effect on the TOR pathway, which controls the target of rapamycin and maintains cell homeostasis. Signalling pathways regulate numerous cellular mechanisms (Dobrenel et al. 2016).

Estève et al. (2009), in order to evaluate the toxic effect of pesticides, Azoxystrobin, Cymoxanil and Diuron also used *S. cerevisiae*, but carried out studies with wild-type strains, to verify the inhibition of the metabolic activity of adenosine triphosphate-ATP production levels, found that the three pesticides analyzed had different toxic effects, due to the nature of the molecule and its concentration, in addition, they found that with the increase in the concentration of the compounds, there was a decrease in ATP in yeast, which is a compound responsible for acting in numerous cellular reactions.

In this study, it can be observed that the toxic action of the 2,4-D compound in relation to FLE yeast cell growth is associated with the exposure time and concentration of the compound. The results obtained in this study corroborate the literature data, which report that depending on the type of substance, the structural nature of the compound and the exposure time, they can cause cytological and genetic alterations. Furthermore, Estève et al. (2009), points out that bioassays using yeasts are considered 96 times faster than a test using *Daphnia Magna* and more sensitive than using *Vibrio fishery*.

In the analysis of images of the cytotoxic action of the 2,4-D pesticide for the inhibition of cellular growth of the yeast Fleischmann®, it can be observed that there was a gradual interaction of the compound's action, inhibiting growth in relation to the times and concentrations analyzed. It was observed that the interaction of the compound with the yeast was more effective in times and

at the highest concentration, possibly under these conditions, the pesticide was more toxic to the yeast (Figure 3). However, the yeast survival against toxic compounds, is closely related to cell wall interaction and inter-genic action, leading to genotypic and phenotypic changes, as an adaptive mechanism to maintain cell integrity and even survival. Possibly, the toxicity mechanism responds differently in *S. cerevisiae*, as observed in this study (Figure 3).

Exposure time (min)	Concentrations (μg L^{-1})			
	Control	2.0	4.0	6.0
30				
60				
90				

Figure 3. Evaluation of images of the cytotoxic action of 2,4-D against the cellular growth of the yeast Fleischmann®. **Source:** Author.

The use of the eukaryotic model can contribute to the understanding of the stressful effect of the presence of pesticides on human health and the environment. Given the fact that several signalling pathways and molecular structures are conserved among eukaryotic organisms, this model can be used to assess the consequences of chemical agents in the expression of fundamental pathways (O'Connor et al. 2013). Therefore, living beings present in the environment can show signs of alterations at the morphological, physiological and genetic level, so such organisms can serve as a study model for detecting the presence of xenobiotic substances. Since the use of microorganisms provides an effective assessment of the toxicity of compounds, in addition to enabling the verification of their respective effects on cells (Mishra et al. 2021).

Studies carried out using *S. cerevisiae* as a bio probe to detect simazine in water and milk were based on the consumption of oxygen by yeast, demonstrating the ability to detect the pesticide in real samples at concentrations up to five times below the legal concentration limits of the European Union (EU), that is, 0.02 parts per billion (ppb) in drinking water and 2 parts per billion (ppb) in cow's milk (Grasso et al. 2018). Furthermore, studies have shown that when *S. cerevisiae* is exposed to xenobiotic compounds, it alters its intracellular mechanisms of repair, detoxification and cellular adaptation, in relation to the action of toxic agents (Dragone et al. 2015).

According to Rumlova & Dolezalova (2012), *S. cerevisiae* has proved to be a model microorganism for several experimental tests with great environmental importance in order to assess possible changes in phenotypic and genotypic expression related to environmental pollutants, especially chemical and agricultural products. Assessing the growth rate is an important form of phenotypic expression evident in *S. cerevisiae* to be measured against changes in toxic compounds. This change shows signs of cell growth inhibition, which may be related to the induction of genes involved in the response to the presence of toxic agents (Braconi et al. 2016).

For the genotoxicity test, it was possible to observe that there was a gradual increase in damage to deoxyribonucleic acid-DNA in relation to concentrations and exposure times compared to 2,4-D for yeast. For the concentration of 2 μg L^{-1} within 30 minutes of exposure to the compound, it was possible to observe a lower rate of DNA damage. However, at the concentration of 6 μg L^{-1} at times of 60 and 90 minutes, it was evident that higher damage rates occurred. Possibly, 2,4-D has directly interacted with the yeast genetic material, triggering changes in the genetic profile of resistance against the compound, evidencing a genotoxic response in relation to 2,4-D (Figure 4).

2,4-D may have other mechanisms of action, as studies report that this compound may exert an inhibitory effect on the TOR pathway, this pathway is linked to cell growth in eukaryotes, regulating the entry of nutrients and protein degradation (Teixeira et al.

2007). Studies based on transcriptome and proteomics analyzes also suggest that the TOR pathway present in yeast regulates the entry of nutrients, thus these analyzes provide an understanding of adaptive response and resistance mechanisms in yeast, increasing knowledge about the toxic effects of agricultural chemicals and pharmacists (Mira et al. 2010).

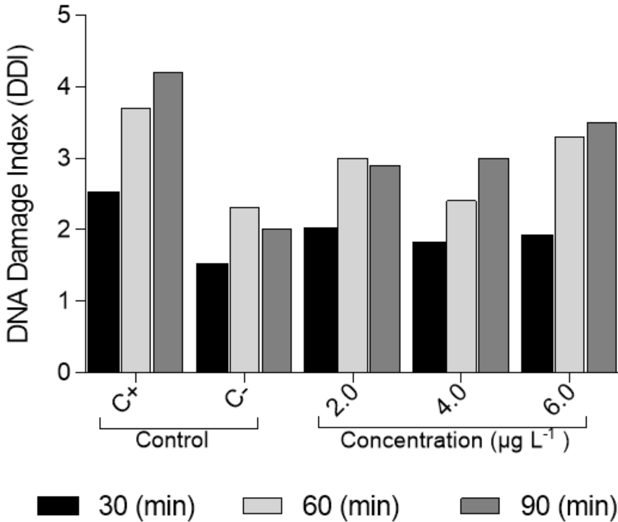

Figure 4. Deoxyribonucleic acid - DNA (DDI) damage index in the yeast Fleischmann® in relation to the concentrations of the 2.4 D pesticide, exposed at different times. Source: Author. C+ (Positive control with hydrogen peroxide 3%), C- (Negative control free from stressing compounds).

Modifications in the gene profile of organisms can provide a quick and sensitive response to the action of a toxic compound, as certain studies report that chemical compounds with analogous toxicological properties can present a characteristic gene expression of a "signature profile" (Smith et al. 2010). In general, cells activate several genes in response to the stressor, in order to protect and repair the damage caused intracellularly by toxic agents. Such a genetic event is quite complex and involves several transcription factors that allow adaptation and resistance under stressful environmental conditions.

Studies carried out by Simões et al. (2003), showed that 13 genes are involved in the regulation of adaptation repression in S. cerevisiae when exposed to 2, 4 D pesticides and these are regulated by the MSN2p and MSN4p genes. However, the adaptation of yeast to this toxic compound involves the unstable and mutable action mediated by these genes. According to Gil et al. (2015), with bioassays using *S. cerevisiae* in exposure to the fungicide pyrimethanil within 2 hours at concentrations of 10 and 45 mg L^{-1}, growth inhibition of 20 to 50% was observed, and a high level of transcription of genes ARG3, ARG5 was also detected, these are related to the toxic action of the studied compound. DNA genomic analysis has proven to be an important tool to verify the simultaneous expressions of numerous genes.

Studies carried out by Liti et al. (2009), using microarray, evidenced alterations in the genome of 70 wild yeasts, such as insertion and deletion of nucleotides, single nucleotides and transposable elements, this technique allows to perform a genome scan. Toxic compounds present in the environment can cause numerous alterations at the cytological and genetic level in living organisms, which may cause inhibition of cell growth, trigger genetic alterations and even be lethal. In this sense, it can be observed that the 2,4-D compound acted on the FLE yeast, inhibiting its growth and inducing DNA damage, therefore, the FLE yeast proved to be an important tool to determine the toxic action of the analyzed compound. However, more studies should be carried out to suggest the use of this yeast as a model to be applied in cytotoxicity and genotoxicity assays.

4. Conclusion

The toxic action of the compound 2,4-D for the yeast FLE occurred at all concentrations analyzed, but within 90 minutes, severe inhibition of cell growth was evident. Yeast FLE proved to be a sensitive bioindicator concerning the toxic action of the analyzed compound.

The comet assay showed a greater amount of DNA damage in the yeast FLE at a concentration of 6 μg L^{-1} against the 2,4-D pesticide. This test is an important methodological tool to assess genotoxicity. The FLE yeast showed potential to be used in environmental analyzes to measure and assess the toxicity degree of the analyzed compound.

Acknowledgment

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001" for MSM and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (311975/2018-6) for CALC.

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