

Original Article

Single and mixture cytogenetic effects evaluation of atrazine and glyphosate herbicides at environmentally relevant concentrations on *Allium cepa* root meristem cells

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Abstract

The mixture of pesticides is employed to obtain other mechanisms of action on target organisms. The herbicides atrazine and glyphosate are used worldwide to control weeds in agricultural crops, and their indiscriminate use results in their constant presence in the environment. In this sense, this study aimed to evaluate the cytotoxic potential, the presence of chromosomal aberrations and nuclear abnormalities, and the frequency of micronuclei of active ingredients and commercial formulations of the herbicides atrazine (AT = 2 and 25 $\mu\text{g L}^{-1}$) and glyphosate (GF = 65 and 160 $\mu\text{g L}^{-1}$) isolated and in mixture (MIX = 2 + 65 and 25 + 160 $\mu\text{g L}^{-1}$). *Allium cepa* root meristem cells were used as the test organism to evaluate environmentally relevant concentrations. Treatments containing the herbicides inhibited the mitotic index (ATAI2: 9.40 ± 1.36 ; GFAI65: 8.58 ± 1.10 ; MIXAI2+65: 6.99 ± 0.57 ; GFAI160: 9.77 ± 1.08 ; ATCOM25: 7.40 ± 1.38 ; GFCOM160: 8.26 ± 0.79) and showed chromosomal aberrations and nuclear abnormalities (ATAI2: 0.85 ± 0.17 ; ATCOM2: 0.97 ± 0.18 ; GFCOM65: 1.00 ± 0.18 ; MIXCOM2+65: 1.09 ± 0.25 ; AITAI25: 0.82 ± 0.17 ; GFAI160: 0.87 ± 0.23 ; MIXAI25+160: 0.95 ± 0.19 ; ATCOM25: 0.67 ± 0.19 ; MIXCOM25+160: 0.80 ± 0.25), indicating the cytotoxic/genotoxic potential of these herbicides. Mutagenic effects were seen in the mixture of the active ingredients of the herbicides at the lowest concentration tested (MIXAI2+65: 0.70 ± 0.57). These results indicate that the isolated herbicides and their mixture directly affect the genetic material. Therefore, we suggest reviewing the acceptable limits of these herbicides in the environment, emphasizing the importance and care required for properly handling these products to minimize environmental and human health risks.

Keywords: Chromosomal abnormalities; Ecotoxicity of mixtures; Micronucleus; Nuclear abnormalities; Pesticides.

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INTRODUCTION

Pesticides are chemicals that control agricultural crop pests to reduce losses and increase productivity (Fatma *et al.*, 2018; Gonçalves and Delabona, 2022). Among pesticides, the class of herbicides is the most used worldwide in weed management (Akash *et al.*, 2022; de Souza *et al.*, 2016; Kuchy *et al.*, 2016). In a global context, Brazil is the largest consumer of pesticides, with the South region corresponding for approximately 25% of total use (IBAMA, 2020). The indiscriminate and widespread application of these compounds causes resistance cases and contributes to the search for new management strategies (Bordin *et al.*, 2018). Therefore, the mixture of different products is an alternative to obtaining other action mechanisms acting on the target plants (Bordin *et al.*, 2021; Patel *et al.*, 2016).

The herbicides atrazine and glyphosate are often reported as the most used worldwide and act on weeds in resistant crops both pre and post-emergence (Gonçalves and Delabona, 2022; Singh *et al.*, 2018). In Brazil, glyphosate was the most sold active ingredient, in tons of active ingredient (TAI), in 2019 (217,592.24 TAI), and atrazine (23,429.38 TAI) occupied the 5th position in the ranking (IBAMA, 2020). Atrazine is part of the chemical group of triazines and acts on the photosynthetic system II, resulting in the death of the target plant (Akash *et al.*, 2022; Stara *et al.*, 2018). The main crops that receive their application are maize, sugar cane, and sorghum (Andrade *et al.*, 2019). Glyphosate, belonging to the chemical group of glycines, acts in the chiquimate pathway inhibiting the enzyme 5-enolpyruvyl-chiquimate-3-phosphate synthetase, interfering in the biosynthesis of essential aromatic amino acids (Brilisaue *et al.*, 2019; Gonçalves and Delabona, 2022). This herbicide is used in soybean, corn, and cotton crops (Bonfleur *et al.*, 2015). However, its presence in the environment is also related to multiple non-agricultural applications (Bridi *et al.*, 2017; Castro Berman *et al.*, 2018).

Due to chemical properties, such as solubility and half-life, these compounds' active ingredients and degradation products are easily transported between different environmental compartments (Brain *et al.*, 2018; de Souza *et al.*, 2016; Lescano *et al.*, 2018). This leads to the frequent presence of these herbicides in the environment, generating risks for non-target species (Carretta *et al.*, 2019; Datta *et al.*, 2018). Environmentally relevant concentrations of atrazine (0.31 - 26.9 $\mu\text{g L}^{-1}$) and glyphosate (0.11 - 427 $\mu\text{g L}^{-1}$) are varied (Alonso *et al.*, 2018; Brovini *et al.*, 2021; Correia *et al.*, 2020; Vieira *et al.*, 2017). In addition, in some cases, the effective concentrations (EC50) that cause toxicity to some indicator organisms are lower and even below the detection limits of the equipment used (Montiel-León *et al.*, 2019; Stara *et al.*, 2018).

Considering that, in addition to the individual application, the association of pesticides is also used, it is necessary to assess the ecotoxicological effects that these mixtures have on non-target organisms since studies on this topic are still scarce (Costa *et al.*, 2022; Gustavsson *et al.*, 2017; Inticher

et al., 2021). As a result, it is not known how these mixtures behave in the ecosystem and whether they are responsible for causing more significant damage compared to their individual applications (Bordin *et al.*, 2021; García-Espiñeira *et al.*, 2018). Likewise, evaluating commercial formulations allows a more realistic estimate of the effects caused, enabling the comparison with the active ingredients to indicate the interference of adjuvants present in commercial products (Ghisi *et al.*, 2016; Nagy *et al.*, 2020).

There is no legislation on permissible limits for the presence of mixtures of compounds in the environment, as this is a recent research topic. Regulations are applied only to individual molecules or compounds. The European Community sets a limit of 0.10 $\mu\text{g L}^{-1}$ for the detection of each individual pesticide and limits the total of individual pesticides detected in aquatic environments to 0.50 $\mu\text{g L}^{-1}$ (European Commission, 1998). In the US, limits are set for both atrazine (3 $\mu\text{g L}^{-1}$) and glyphosate (700 $\mu\text{g L}^{-1}$) in waters intended for human consumption (USEPA, 2009). In Brazil, there are also water potability limits imposed by legislation for atrazine (2 $\mu\text{g L}^{-1}$) and glyphosate (500 $\mu\text{g L}^{-1}$) (Brazil, 2021), both of which are higher than those established by the European Community. Furthermore, according to Brazilian legislation, 2 $\mu\text{g L}^{-1}$ for atrazine and 65 $\mu\text{g L}^{-1}$ for glyphosate are permitted concentrations in superficial waters, which are destined for supply for human consumption after simplified treatment (Brasil, 2005).

Due to characteristics such as proliferation kinetics, high sensitivity, low cost, and easy handling, the ecotoxicity assay using *Allium cepa* as a bioindicator is an excellent model for the evaluation of many pollutants in varying concentrations (Gallego and Olivero-Verbel, 2021; Kuchy *et al.*, 2016; Sheikh *et al.*, 2020). Furthermore, due to the similarity in chromosome morphology with mammals, this plant can potentially evaluate the cytogenotoxic effects of substances that may affect the genetic material (Mercado and Caleño, 2020). Among the parameters observed when performing the test using *Allium cepa* as a bioindicator are the changes observed in the roots, such as color, texture, thickness, and length (Datta *et al.*, 2018). In addition, microscopic parameters, such as the appearance of aberrations and irregularities in the structure of chromosomes, indicate disturbances in the process of cell division, resulting in changes in cell content (Mercado and Caleño, 2020). Through this test, it is possible to determine parameters such as cytotoxicity, which is related to the mitotic index; genotoxicity, which refers to the chromosomal aberrations and nuclear abnormalities; and mutagenicity, characterized by the presence of micronuclei in the cells (Felisbino *et al.*, 2018; Lima *et al.*, 2019; Mahapatra *et al.*, 2019).

Studies that evaluate the effects of pesticides mixture are scarce, and as there are no limits imposed by legislation, their application and, consequently, their release into the environment is inevitable and occurs increasingly. Therefore, this study aimed to investigate the cytogenetic effects of the active ingredients and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture,

in environmentally relevant concentrations, using *Allium cepa* root meristem as a test organism. Considering that the mixture of compounds can cause additive, antagonistic, and synergistic effects (Golin *et al.*, 2022; Inticher *et al.*, 2021), we hypothesized that the mixture of herbicides would be responsible for potentiating the toxic effects on the biological model and that the commercial formulations will be more toxic compared to their respective active ingredients.

MATERIALS AND METHODS

Test solutions

Stock solutions of the active ingredients for atrazine (CAS No. 1912-24-9, Sigma-Aldrich®, purity $\geq 98\%$) and glyphosate (CAS No. 1071-83-6, Sigma-Aldrich®, purity $\geq 98\%$) were prepared in purified water (with a reverse osmosis system), in concentrations of 20 and 500 mg L⁻¹, respectively. The stock solutions of the commercial formulations of the herbicides atrazine (commercial formulation containing 40% of the active ingredient atrazine)

and glyphosate (commercial formulation containing 48% of the active ingredient glyphosate) were prepared based on the amount of the active ingredient at a concentration of 100 mg L⁻¹ in purified water (with a reverse osmosis system).

The final solutions were prepared by diluting stock solutions in purified water (with a reverse osmosis system) according to the concentrations evaluated. The concentrations of 2 and 25 $\mu\text{g L}^{-1}$ were evaluated for atrazine (AT), and 65 and 160 $\mu\text{g L}^{-1}$ for glyphosate (GF), in addition to the mixture (MIX) of the smaller (2 + 65 $\mu\text{g L}^{-1}$) and higher (25 + 160 $\mu\text{g L}^{-1}$) concentrations of atrazine and glyphosate, respectively. These concentrations were evaluated for both active ingredients (AI) and commercial formulations (COM) and were defined based on the analysis of studies that determined the presence of these herbicides in environmental samples (Supplementary Figure 1) (Alonso *et al.*, 2018; Carles *et al.*, 2019; Chen *et al.*, 2019; Mahler *et al.*, 2017; Montiel-León *et al.*, 2019; Poiger *et al.*, 2017; Vieira *et al.*, 2017).

Negative (NC) and positive (PC) controls were included as purified water (with a reverse osmosis system) or the mutagenic agent methyl methanesulfonate (CAS No. 66-27-3, Sigma-Aldrich®) (MMS - 10 mg L⁻¹). All chemicals used were of analytical grade.

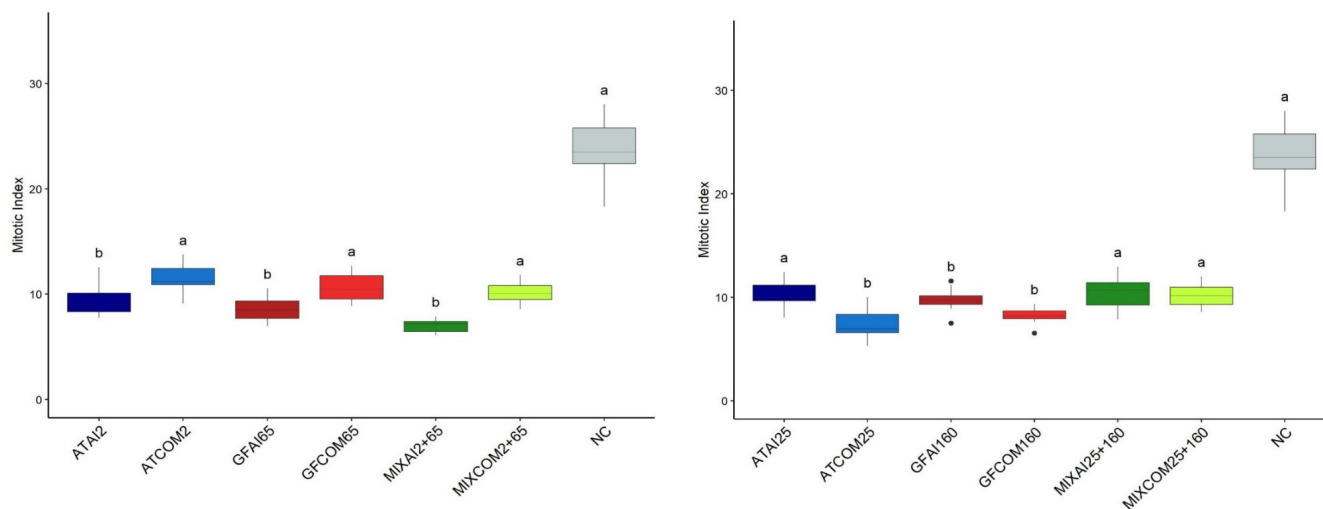


Figure 1: Mitotic index obtained through the analysis of meristematic cells of *Allium cepa* roots exposed to active ingredients and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture.

ATAI: atrazine active ingredient; ATCOM: atrazine commercial formulation; GFAl: glyphosate active ingredient; GFCOM: glyphosate commercial formulation; MIXAI: mixture of active ingredients (ATAI+GFAl); MIXCOM: mixture of commercial formulations (ATCOM+GFCOM); NC: negative control (purified water with a reverse osmose system).

Data expressed in percentage (%). 5000 cells analyzed per treatment. Different letters indicate statistical difference ($p < 0.05$) related to NC, according to the Kruskal-Wallis followed by Dunn.

Bioassays using *Allium cepa*

The assay using *Allium cepa* as a bioindicator used the methodology described by Felisbino *et al.* (2018) and Leme and Marin-Morales (2008). Twenty commercial seeds of the Baia periforme variety, with a germination index of 94%, free of pesticides, were randomly distributed in Petri dishes on filter paper soaked in 4 mL of the test solutions. The assay was performed in duplicate (2n = 40). The plates were incubated for 96 hours at 25 °C and protected from light. Roots with approximately 2 cm in length (96 h) were collected, fixed in ethanol:acetic acid (3:1 – v/v), and stored at 4 °C until use (Felisbino *et al.*, 2018). The hydrolysis process occurred for 10 minutes with the addition of the roots in a solution of HCl 1 mol L⁻¹ previously stabilized in a water bath at 60 °C. The hydrolyzed roots were transferred to flasks containing Schiff's reagent, where they remained for 2 hours protected from light (Felisbino *et al.*, 2018; Leme and Marin-Morales, 2008).

The meristematic region of *Allium cepa* roots was used to prepare 10 slides per treatment. A drop of acetic carmine solution (2%) was added to the meristematic regions, which were covered with a coverslip and carefully crushed (Leme and Marin-Morales, 2008). The slides were fixed using liquid nitrogen and Canadian balsam, and the reading was performed under an optical microscope (400x). Approximately 500 cells per slide were observed, totaling approximately 5000 cells per treatment. Cytotoxicity endpoints were verified through the mitotic index (MI) (Equation 1), which comprised the number of dividing cells (prophase, metaphase, anaphase, and telophase). Genotoxicity endpoints were determined using different types of chromosomal aberration (CA), nuclear abnormalities (NA), and micronuclei (MN). CA and NA were classified into aneugenic (chromosomal adhesion, delays and losses, and nuclear buds) and clastogenic (chromosomal breaks and bridges) to verify the genotoxic mode of action of the tested herbicides (Equation 2). The micronuclei frequency (MN) was determined from Equation 3.

$$MI = \frac{\text{Dividing cells}}{\text{Observed cells}} \times 100 \quad (\text{Equation 1})$$

$$CA + NA = \frac{\text{Chromosomal aberration cells} + \text{nuclear abnormalities cells}}{\text{Observed cells}} \times 100 \quad (\text{Equation 2})$$

$$MN = \frac{\text{Micronucleated cells}}{\text{Observed cells}} \times 100 \quad (\text{Equation 3})$$

Statistical analysis

Data were analyzed with R statistic software. All data were checked for normality (Shapiro-Wilk test) and homoscedasticity (Levene test) followed by Kruskal-Wallis test followed by Dunn's test with a 95% confidence interval ($p < 0.05$).

RESULTS AND DISCUSSION

Mitotic Index

The treatments containing the herbicides atrazine and glyphosate (ATAI2, GFAI65, MIXAI2+65, ATCOM25, GFAI160, and GFCOM160) significantly inhibited ($p < 0.05$) the cell division process (Figure 1) related to NC. Furthermore, the treatments with the active ingredients at the lowest concentration tested (ATAI2, GFAI65, and MIXAI2+65) were responsible for a significantly higher inhibition of the mitotic index ($p < 0.05$) than their respective commercial formulations (ATCOM2, GFCOM65, and MIXCOM2+65). The positive control (methyl methanesulfonate - MMS 10 mg L⁻¹) was used to validate the assay. Therefore, our hypotheses were not confirmed for this parameter because the active principles isolated and their mixture at the lowest concentration tested significantly reduced cell division.

Were identified cells in interphase and the process of mitotic cell division: prophase, metaphase, anaphase, and telophase. When comparing the cell data in the prophase phase (Table 1) to other phases of the cell cycle (metaphase, anaphase, and telophase), it is noticed that the highest value is presented for cells in prophase, demonstrating that from these phases, there is resistance to normally follow the cell cycle.

When evaluating the commercial formulation with glyphosate as an active ingredient, the mitotic index decreased at a concentration of 160 µg L⁻¹ (8.26 ± 0.79) compared to a concentration of 65 µg L⁻¹ (10.61 ± 1.28). The same was observed by Mercado and Caleño (2020) when they used *A. cepa* as a bioindicator and noticed that the mitotic index decreased when the concentrations of glyphosate, a commercial product, increased since the highest concentration of glyphosate evaluated by the authors (30 mg L⁻¹) there was a 90.8% inhibition of mitosis. Exposure of *A. cepa* to the herbicide atrazine (15, 30, and 60 mg L⁻¹) also resulted in the inhibition of MI in a dose-dependent manner (Srivastava and Mishra, 2009). However, these studies evaluated concentrations much higher than those in the environment. Hence, our study presents important results on evaluating concentrations of environmental relevance, especially on the mixture of two of the most widely used herbicides in the world.

The trend of the results indicates that the mitotic index was affected by both herbicides and, equally, by the mixture of these. The significant reduction ($p < 0.05$) of MI related to negative control, denotes the inhibition of cell proliferation caused by chemical compounds, representing the direct alteration of the genetic material of *Allium cepa* (Inticher *et al.*, 2021; Silveira *et al.*, 2016). The mitotic index estimates the frequency of cell division and the proportion of cells in the different mitotic phases (Mercado and Caleño, 2020; Rodríguez *et al.*, 2015). Pesticide performance disrupts the normal development of the mitotic cycle, preventing the cycle

of mitosis during interphase, and this may occur due to the inhibition of DNA/protein synthesis (Kuchy *et al.*, 2016). The observed decrease in mitotic activity probably indicates a mitodepressive effect, that is, the normal development of

mitosis was affected, preventing cells from moving to the next phases, and the mitotic cycle was blocked during interphase (Silveira *et al.*, 2017).

Table 1: Changes related to the mitotic index, obtained through the analysis of meristematic cells of *Allium cepa* roots exposed to the active ingredients and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture.

Group	Total cells	Prophase	Metaphase	Anaphase	Telophase	MI
		M ± sd	M ± sd	M ± sd	M ± sd	M ± sd
Negative Control	5365	19.07 ± 3.25a	2.04 ± 0.76*	0.82 ± 0.46*	1.53 ± 0.70a*	23.46 ± 3.01a
Atrazine AI 2 µg L ⁻¹	5130	4.74 ± 0.68b	2.18 ± 0.80	1.48 ± 0.24*	1.00 ± 0.46*	9.40 ± 1.36b
Glyphosate AI 65 µg L ⁻¹	5258	4.12 ± 0.75b	1.89 ± 0.74*	1.13 ± 0.54*	1.43 ± 0.49*	8.58 ± 1.10b
Mixture AI 2+65 µg L ⁻¹	5114	3.72 ± 0.87b	1.70 ± 0.60	0.89 ± 0.28*	0.68 ± 0.32*	6.99 ± 0.57b
Atrazine COM 2 µg L ⁻¹	5098	6.25 ± 1.17	2.53 ± 0.83	1.37 ± 0.47*	1.02 ± 0.53*	11.54 ± 1.25
Glyphosate COM 65 µg L ⁻¹	5254	5.54 ± 0.69	2.47 ± 0.66	1.55 ± 0.53*	1.05 ± 0.37*	10.61 ± 1.28
Mixture COM 2+65 µg L ⁻¹	5184	4.89 ± 0.81b	2.68 ± 0.65	1.50 ± 0.44*	1.09 ± 0.52*	10.17 ± 0.98
Atrazine AI 25 µg L ⁻¹	5155	5.69 ± 1.04	2.18 ± 0.60	1.08 ± 0.43*	1.40 ± 0.65*	10.35 ± 1.34
Glyphosate AI 160 µg L ⁻¹	4996	4.96 ± 0.56b	2.54 ± 0.77	1.16 ± 0.52*	1.11 ± 0.52*	9.77 ± 1.08b
Mixture AI 25+160 µg L ⁻¹	5033	6.01 ± 1.15	2.45 ± 1.37	0.90 ± 0.51*	1.08 ± 0.56*	10.44 ± 1.57
Atrazine COM 25 µg L ⁻¹	5016	4.50 ± 0.78b	1.45 ± 0.93	0.71 ± 0.33*	0.74 ± 0.35*	7.40 ± 1.38b
Glyphosate COM 160 µg L ⁻¹	5056	4.70 ± 0.46b	2.05 ± 0.70	0.91 ± 0.42*	0.60 ± 0.37b*	8.26 ± 0.79b
Mixture COM 25+160 µg L ⁻¹	5244	5.22 ± 0.54	2.80 ± 0.52	1.32 ± 0.37*	0.84 ± 0.38*	10.17 ± 1.02
Methyl methanesulfonate 10 mg L ⁻¹	5347	23.02 ± 7.92	1.94 ± 0.92	0.80 ± 0.49	1.02 ± 0.51	26.79 ± 8.35

AI: active ingredient, COM: commercial formulation. Data expressed in percentage (%) and mean (M) ± standard deviation (sd). 5000 cells analyzed per treatment.

Different letters indicate statistical difference ($p < 0.05$) related to NC. Asterisk indicates statistical difference ($p < 0.05$) between prophase and the other phases of cell division.

Complex mixtures of chemical products, such as herbicides, are present in the environment in different concentrations (ng-mg/L), and the interactions that may occur between these chemical products can modulate the effects on biological systems (Felisbino *et al.*, 2018; Oliveira *et al.*, 2021), as verified in other studies of our group (Bordin *et al.*, 2022; Bordin *et al.*, 2023). Nevertheless, the cytotoxicity of herbicide mixtures is not widely reported in the literature (Bianchi *et al.*, 2016; Costa *et al.*, 2022; Fatma *et al.*, 2018; Felisbino *et al.*, 2018; Sheikh *et al.*, 2020), making it difficult to interpret the possible interactive effects between the compounds. The mixture of atrazine with a fungicide (difenoconazole) and an insecticide (fipronil) has already been evaluated and resulted in MI inhibition and CA induction, probably due to the maximization of the individual effects of each compound (Inticher *et al.*, 2021).

Cytotoxic effects can be caused by oxidative stress, meaning that when macromolecules such as DNA are damaged, the cell cycle is interrupted so that repair systems can be activated before resuming cellular progression. However, cell death is triggered when the damage is severe and cannot repair (Bianchi *et al.*, 2016; Oliveira *et al.*, 2021). Therefore, the observed reduction in MI suggests that cell cycle disruption or delay and induction of cellular death occurred as a response to the cytotoxic action of both herbicides and their mixture.

Considering the use of selective herbicides, the undesired effects caused to non-target species (e.g., *Allium cepa*) can interfere with natural processes resulting in increasing cases of pesticide resistance, induction of morphological changes, and the extinction of more sensitive species (de Souza *et al.*, 2016; Lukaszewicz *et al.*, 2019).

Chromosomal aberrations and nuclear abnormalities

When evaluating the frequency of chromosomal aberrations (CA) and nuclear abnormalities (NA), was verified a significant increase ($p < 0.05$) related to the negative control for ATAI2, ATCOM2, GFCOM65, MIXCOM2+65, ATAI25, ATCOM25, GF AI160, MIXAI25+160, and a significant increase ($p < 0.05$) for treatments with commercial formulations (GFCOM65 and MIXCOM2+65) in relation to their respective active ingredients (GF AI65 and MIXAI2+65) (Figure 2). Our first hypothesis was not supported for this parameter, considering that both the herbicides isolated and their mixture showed a significant increase in CA and NA. But our second hypothesis was supported, given that the commercial formulation of glyphosate and the mixture of the commercial formulations showed an increase in CA and NA compared to the respective active ingredients.

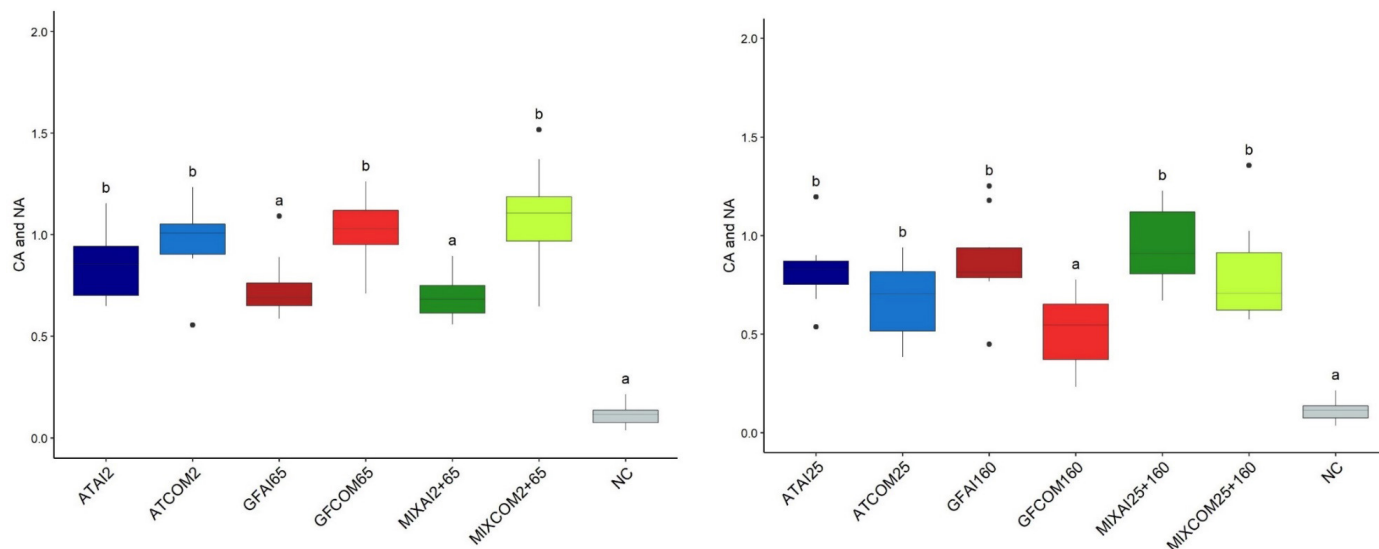


Figure 2: Chromosomal aberrations and nuclear abnormalities obtained through the analysis of meristematic cells of *Allium cepa* roots exposed to active ingredients and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture.

ATAI: atrazine active ingredient; ATCOM: atrazine commercial formulation; GFAI: glyphosate active ingredient; GFCOM: glyphosate commercial formulation; MIXAI: mixture of active ingredients (ATAI+GFAI); MIXCOM: mixture of commercial formulations (ATCOM+GFCOM); NC: negative control (purified water with a reverse osmose system).

Data expressed in percentage (%). 5000 cells analyzed per treatment. Different letters indicate statistical difference ($p < 0.05$) related to NC, according to the Kruskal-Wallis followed by Dunn.

The mitotic index and the frequency of chromosomal aberrations and nuclear abnormalities decreased in the groups containing the commercial formulation of atrazine and glyphosate in the highest concentration compared to the same groups in the lowest concentration evaluated. This fact may be linked to the concentration used since ATCOM25 and GFCOM160 induced a lower frequency of aberrant cells than ATCOM2 and GFCOM65. This response may be related to inhibition of the mitotic index, considering that approximately 92% and 91% of the cells of the roots of *A. cepa* presented in interphase for the groups exposed to ATCOM25 and GFCOM160, respectively. While for the ATCOM2 and GFCOM65 groups, the interphase cells corresponded to 88% and 89%, respectively.

The decrease in MI along with the decrease in AC was observed by Bianchi et al. (2016) when evaluating the effects of the herbicide sulfentrazone and by Liman et al. (2015) when evaluating the effects of the herbicide imazethapyr on meristematic cells of *A. cepa*. In this sense, the herbicides evaluated in our study may have led to an increase in the

induction of cytotoxic compounds, which interfere with the normal progression of the cell cycle. In response to some damage, there is usually a delay in the mitotic cycle, resulting in a low MI due to delays arising from the repair processes of injuries induced by herbicides (Bianchi et al., 2016). Therefore, we suggest that the frequencies of aberrant cells are also reduced due to the MI reduction.

Regarding genetic damage, was observed aneugenic (adhesions, nuclear sprouts, chromosome delays, and losses) and clastogenic (chromosome bridges and fragments) chromosomal aberrations (Silveira et al., 2017; Souza et al., 2020). Adhesions, bridges, delays, and nuclear buds were chromosomal aberrations and nuclear abnormalities that showed significant difference ($p < 0.05$) when treatments with the herbicides atrazine and glyphosate were compared to the negative control (Table 2). Therefore, both herbicides and their mixture are genotoxic to this non-target plant.

Table 2: Chromosomal aberrations and nuclear abnormalities observed in meristematic cells of *Allium cepa* roots exposed to the active ingredients and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture.

Group	Adhesions M ± sd	Bridges M ± sd	Delays M ± sd	Fragments M ± sd	Losses M ± sd	Nuclear buds M ± sd	CA M ± sd
Negative Control	0.02 ± 0.06	0.22 ± 0.16	0.28 ± 0.15	0.02 ± 0.06	0.00 ± 0.00	0.02 ± 0.05	0.11 ± 0.06
Atrazine AI 2 µg L ⁻¹	1.18 ± 0.41*	0.93 ± 0.31*	0.97 ± 0.43*	0.02 ± 0.06	0.00 ± 0.00	1.17 ± 0.37*	0.85 ± 0.17*
Glyphosate AI 65 µg L ⁻¹	1.13 ± 0.45*	0.88 ± 0.54	0.94 ± 0.31	0.00 ± 0.00	0.04 ± 0.08	0.70 ± 0.21	0.74 ± 0.15
Mixture AI 2+65 µg L ⁻¹	1.17 ± 0.18*	0.51 ± 0.22	0.63 ± 0.43	0.00 ± 0.00	0.02 ± 0.06	1.13 ± 0.22*	0.69 ± 0.10
Atrazine COM 2 µg L ⁻¹	1.23 ± 0.37*	0.86 ± 0.44*	1.14 ± 0.45*	0.00 ± 0.00	0.08 ± 0.14	1.55 ± 0.61*	0.97 ± 0.18*
Glyphosate COM 65 µg L ⁻¹	1.20 ± 0.41*	1.27 ± 0.50*	1.07 ± 0.48*	0.04 ± 0.12	0.08 ± 0.10	1.37 ± 0.35*	1.00 ± 0.18*
Mixture COM 2+65 µg L ⁻¹	1.41 ± 0.39*	1.35 ± 0.61*	1.36 ± 0.41*	0.00 ± 0.00	0.04 ± 0.12	1.29 ± 0.57*	1.09 ± 0.25*
Atrazine AI 25 µg L ⁻¹	1.04 ± 0.54*	0.94 ± 0.34*	0.92 ± 0.40*	0.02 ± 0.06	0.00 ± 0.00	1.19 ± 0.61*	0.82 ± 0.17*
Glyphosate AI 160 µg L ⁻¹	1.46 ± 0.38*	0.93 ± 0.62*	0.87 ± 0.35*	0.06 ± 0.09	0.00 ± 0.00	1.04 ± 0.41*	0.87 ± 0.23*
Mixture AI 25+160 µg L ⁻¹	1.30 ± 0.50*	0.74 ± 0.53	0.94 ± 0.36*	0.13 ± 0.20	0.04 ± 0.08	1.61 ± 0.38*	0.95 ± 0.19*
Atrazine COM 25 µg L ⁻¹	1.01 ± 0.41*	0.56 ± 0.38	0.55 ± 0.34	0.03 ± 0.09	0.02 ± 0.05	1.16 ± 0.46*	0.67 ± 0.19*
Glyphosate COM 160 µg L ⁻¹	0.89 ± 0.36*	0.65 ± 0.32	0.49 ± 0.37	0.00 ± 0.00	0.02 ± 0.06	0.54 ± 0.25	0.52 ± 0.17
Mixture COM 25+160 µg L ⁻¹	1.30 ± 0.57*	0.76 ± 0.42	0.78 ± 0.36	0.00 ± 0.00	0.00 ± 0.00	1.15 ± 0.53*	0.80 ± 0.25*
Methyl methanesulfonate 10 mg L ⁻¹	0.44 ± 0.33	0.11 ± 0.12	0.64 ± 0.49	1.00 ± 0.75*	0.11 ± 0.16	0.45 ± 0.24	0.55 ± 0.30

AI: active ingredient, COM: commercial formulation. Data expressed in frequency (%) and mean (M) ± standard deviation (sd). 5000 cells analyzed per treatment.

* Significant difference related to NC ($p < 0.05$), according to the Kruskal-Wallis test, followed by Dunn.

The most common were chromosomal adhesions and nuclear buds, followed by delays and chromosomal bridges. However, chromosomal fragments and losses were not as frequent. Thus, our results indicate the cytotoxic/genotoxic potential of these herbicides (Figure 1 and Table 2). In our study, both the herbicides atrazine and glyphosate isolated and their mixture acted as genotoxic by both aneugenic (adhesion, sprouting, and delay) and clastogenic (bridging) mechanisms of action. These mechanisms of action have already been reported for atrazine and glyphosate, highlighting as clastogenic effect the chromosomal bridges and as aneugenic effect the chromosomal adhesions (Felisbino *et al.*, 2018; Mercado and Caleño, 2020).

According to de Souza *et al.* (2016), herbicides can decrease the mitotic index and, together, induce mitotic chromosomal aberrations in *A. cepa*, both effects being observed in this study. Grillo *et al.* (2012) also verified the decrease in the mitotic index and the induction of chromosomal aberrations in *A. cepa* cells treated with active ingredient atrazine (1, 10 and 100 mg L⁻¹), and concluded that these results indicate the cytotoxic potential this herbicide. High chromosome aberration frequency, with chromosomal adhesions and bridges being the most prevalent, have already been reported for exposure of *A. cepa* to atrazine in a higher concentration than those present in the environment (3 g L⁻¹) (Silveira *et al.*, 2017). Increased chromosomal changes in *A. cepa* cells were also verified after exposure to glyphosate herbicide (5, 10, 15, 25, and 30 mg L⁻¹), indicating its direct action on the genetic material and its genotoxic potential (Mercado and Caleño, 2020).

After evaluating the effects of mixtures of the same herbicides evaluated in our study (glyphosate and atrazine), Roustan *et al.* (2014) demonstrated that these pesticides establish cytogenetic impacts in mammals, and that conditions such as solar irradiation enhance the clastogenic and aneugenic effects of pesticides. The authors also observed that the pesticide mixture had genotoxic properties in concentrations lower than those of the individual molecules. Similar to our study, Mahapatra *et al.* (2019) observed effects such as the reduction of the mitotic index and the presence of chromosomal aberrations, where metaphases with adhesions and anaphasic bridges were the most frequent aberrations when the effects of two fungicides (tricyclazole and thiabendazole) and two insecticides (Plethora and Slash-360) were tested in high concentrations in *Trigonella foenum - graecum*.

Changes in the structure of chromosomes or in their total number can occur when root meristem cells of *Allium cepa* are exposed to chemical agents (de Souza *et al.*, 2016; Magdaleno *et al.*, 2015). This test system evaluates chromosomal aberrations and nuclear abnormalities, including changes in genetic material (Lukaszewicz *et al.*, 2019; Rodríguez *et al.*, 2015; Sabeen *et al.*, 2020). Inhibition of synthesis and replication, as well as DNA breaks, are primarily responsible for chromosomal changes, which can be spontaneous or induced by external agents (de Souza *et al.*, 2016; Rodríguez *et al.*, 2015). NA are morphological changes in the nucleus that occur due to the activation of cell repair genes, which act to eliminate the action of xenobiotics, but result in mitotic errors (Inticher *et al.*, 2021).

The large presence of chromosomal adhesions can result in cell death, since they enter the division process, but do not end the mitotic cycle, as the adhesion cannot be repaired (Lima *et al.*, 2019; Liman *et al.*, 2015; Silveira *et al.*, 2017). Chromosomal adhesions may result from increased chromosomal contraction due to failures in the spindle depolymerization process (Felisbino *et al.*, 2018; Fernandes *et al.*, 2009). The nuclear buds, which have also been observed frequently, originate from the delayed chromosomes surrounded by the cell membrane, which form a compartment partially separated from the nucleus (Serrano-García and Montero-Montoya, 2001). However, according to Fernandes *et al.* (2009), this change does not represent an aberration, as it can still assume its characteristic morphology. Chromosomal delays occur due to the failure of chromosomes to move to either pole, and this aberration is responsible for the increased risk of aneuploidy (Liman *et al.*, 2015; Rodríguez *et al.*, 2015). Chromosomal bridges occur due to structural changes between sister chromatids or between different chromosomes that result from terminal breaks and deletions (Bianchi *et al.*, 2016; Liman *et al.*, 2015; Silveira *et al.*, 2017). At the end of cell division, chromosomal bridges can break and give rise to chromosomal fragments that can become MN in daughter cells (Bianchi *et al.*, 2016; Felisbino *et al.*, 2018; Leme and Marin-Morales, 2008).

The effects of the active ingredients and their mechanisms of action are well determined. Thus, studies that address commercial products are important, as the evaluation of the ecotoxicological effects of pesticide formulations needs to be better documented, as is the evaluation of pesticide mixtures (de Souza *et al.*, 2016). Few studies have evaluated the mixture of pesticides so far due to the difficulty of evaluating

and interpreting the results, as it is a challenge to identify and attribute the effects to the different molecules present in the mixture (Bianchi *et al.*, 2016; Fatma *et al.*, 2018; Felisbino *et al.*, 2018; Kuchy *et al.*, 2016; Patel *et al.*, 2016; Sheikh *et al.*, 2020). The results verified may be caused by the presence of adjuvants in the formulations, which can result in the induction of the genotoxic effect when combining the herbicides, as was the case of the mixture of atrazine and glyphosate (Bianchi *et al.*, 2016; Felisbino *et al.*, 2018; Gustavsson *et al.*, 2017; Nagy *et al.*, 2020). However, it may also occur due to the mixture that already exists in commercial products, due to the presence of other molecules, which can modify the toxicity of herbicides (Magdaleno *et al.*, 2015). Therefore, when studying the association of different active ingredients, unexpected results can be obtained, considering the additive or inhibitory, synergistic, and potentiation interactions that can occur between them (Gustavsson *et al.*, 2017; Nagy *et al.*, 2020).

Micronucleus frequency

For the frequency of micronuclei, the only group that showed a significant increase ($p < 0.05$) was the mixture of active ingredients at the lowest concentration evaluated (MIXAI2+65) (Figure 3). Thus, our hypothesis was corroborated for this parameter, suggesting that the mixture of herbicides may be causing a possible synergistic effect and being responsible for mutagenic effects in *A. cepa* cells. However, the second hypothesis was not confirmed as this effect was not observed for the mixture of the commercial formulations.

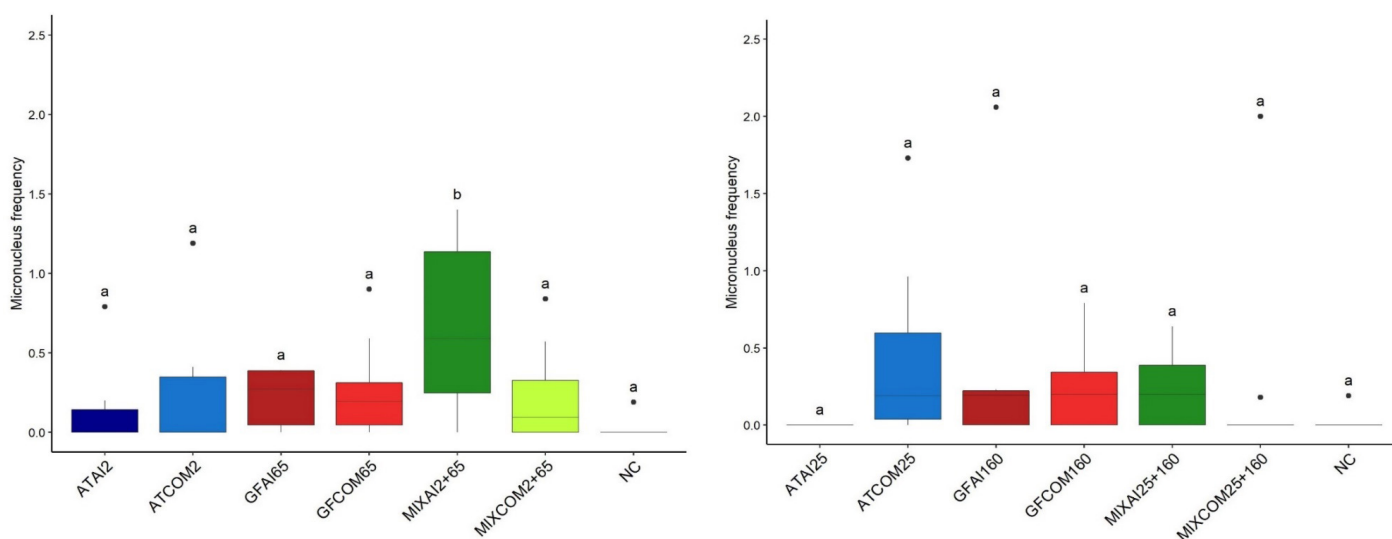


Figure 3: Mutagenic alterations obtained through the analysis of *Allium cepa* roots meristematic cells exposed to the active ingredients and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture.

ATAI: atrazine active ingredient; ATCOM: atrazine commercial formulation; GFAl: glyphosate active ingredient; GFCOM: glyphosate commercial formulation; MIXAI: mixture of active ingredients (ATAI+GFAl); MIXCOM: mixture of commercial formulations (ATCOM+GFCOM); NC: negative control (purified water with a reverse osmose system).

Data expressed in percentage (%). 5000 cells analyzed per treatment. Different letters indicate statistical difference ($p < 0.05$) related to NC, according to the Kruskal-Wallis followed by Dunn.

Micronuclei originated from chromosomal fragments or disturbances in the mitotic process during cell division (Ghisi *et al.*, 2016; Magdaleno *et al.*, 2015) and were not frequently observed in our study. This fact may have occurred due to the low concentrations evaluated. In addition, the low mitotic index resulting from the inhibition of cell proliferation may be related to a lower frequency of micronuclei (Silveira *et al.*, 2016). However, our results do not support this suggestion, as the highest frequency of micronuclei was observed in the group with the lowest mitotic index.

The mixture of the active ingredients of the herbicides presented a greater mutagenic effect when compared to the herbicide atrazine in its isolated form. Similar to that reported by Felisbino *et al.* (2018) when evaluating mesotrione associated with atrazine in environmentally relevant concentrations (1.5; 6.25; 25; 100, and 400 $\mu\text{g L}^{-1}$), and the increase in genotoxicity and the frequency of micronuclei were superior to the effects of the same isolates, indicating that the use of mesotrione in mixture with other genotoxic herbicides acts as co-mutagen. As was reported in our study, cells with micronuclei were not significant when Bianchi *et al.* (2016) evaluated the pesticides imidacloprid (0.036; 0.36, and 3.6 g L^{-1}) and sulfentrazone (0.06; 0.6, and 1.2 g L^{-1}) and their mixtures. The mixture of these pesticides resulted in cytotoxic and genotoxic effects, reducing the cell division index, and in the same way, as presented in our study, adhesions were the main chromosome aberration observed.

Ghisi *et al.* (2016) state that in comparing the active principle and commercial glyphosate formulations, the greatest micronuclei formation is observed when the commercial product is tested. Our results obtained this response when the highest concentration of glyphosate was evaluated. However, the opposite occurred when the lowest concentration was evaluated. In the study by Rodríguez *et al.* (2015), the frequency of micronuclei was dependent on the concentration, as it presented a statistical difference ($p < 0.05$) for the concentration that simulated the indiscriminate use of this pesticide (80 mg m^2 - twice the recommended concentration). Likewise, our results differ from those presented by the authors, as they indicate that the highest frequency of micronuclei occurred in the group exposed to the mixture of the lowest concentration of herbicides.

We highlight the scarcity of studies that address the mixture of herbicides and suggest that, based on our results, studies in this area should be developed to stimulate the proper management of these contaminants. It is known that regulatory agencies usually require toxicological evaluation of active ingredients or isolated finished products to register chemicals. This limitation should be explored for the environmental management of product launches. This is because, in the real environmental scenario, the simultaneous presence of several contaminants occurs, which, when interacting, can alter the predicted toxicity of the isolated compound (Felisbino *et al.*, 2018; Inticher *et al.*, 2021; Oliveira *et al.*, 2021).

Based on the limits set for these herbicides in aquatic

environments, the concentrations evaluated in this study exceed the limit allowed by the European Community, which is the safest. Was verified the inhibition of the mitotic index and induction of nuclear abnormalities and chromosomal aberrations, and the mixture of atrazine and glyphosate presented the mutagenic effect at the lowest concentration tested (MIXAI2+65). Therefore, more studies involving other test organisms and other methodologies and test repetitions are needed to change or revise Brazilian and US legislation on the use of pesticides. More severe restrictions on releasing of these pesticides to the environment are fundamental, considering that the long-term environmental impacts can be severe.

Therefore, considering the current environmental scenario, several molecules simultaneously form complex mixtures, which may be responsible for different effects on bioindicator organisms (Felisbino *et al.*, 2018). These compounds can interact with each other, changing ecotoxicological effects through synergism, potentiation, or inhibition, and mixtures are often reported to cause potential effects when compared to isolated molecules (Goujon *et al.*, 2014; Nagy *et al.*, 2020). In addition, pesticide residues constantly reach the environment and cause serious concerns, as their long-term adverse effects are unknown (Kuchy *et al.*, 2016).

CONCLUSION

The results obtained in this study demonstrated that the active ingredients and commercial formulations of the herbicides evaluated in all the environmentally relevant concentrations, both in their isolated form and in mixture, present a cytotoxic/genotoxic effect, presenting a potential environmental risk. The mixture of herbicide active ingredients, at the lowest concentration tested, was the group that showed a significant increase ($p < 0.05$) of micronuclei related to the negative control, highlighting its mutagenic potential.

However, considering the need to use pesticides, the importance of developing less toxic formulations that can contribute to environmentally safe productivity is perceived. The relevance of evaluating the responses presented by low concentrations is highlighted, as well as by the mixture of pesticides, considering that in the environment, a great variety of these products in different concentrations is found. Still, in view of the effects presented by the two herbicides and their mixture, the necessary attention for the proper handling of these products is emphasized, aiming to minimize environmental and human health risks.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CREDIT AUTHOR STATEMENT

ERB: Conceptualization, Methodology, Writing, Data Interpretation, Original draft preparation, Investigation.
FS: Methodology, Visualization. **YM:** Methodology, Visualization. **AMF:** Visualization, Reviewing, Supervision.
WAR: Visualization, Reviewing, Supervision.

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