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*Essay*

# Efects of a S-Metolachlor Based Herbicide on Two Plant Models: Zea Mays L. And *Lactuca Sativa L.*

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**ABSTRACT:** Brazil is the number-one country in pesticide consumption, and corn is the second most cultivated crop in the country. Chemical control of weeds associated with corn cultivation is performed by application of herbicides with pre- and postemergence action, such as S-metolachlor. Currently, the toxicity of herbicides is a task of great concern. In this regard, the present study aimed to evaluate the effects of an S-metolachlor-based herbicide through bioassays with the plant model *Lactuca sativa* L. (lettuce) and *Zea mays* L. (maize). The herbicidal test solutions containing 7.5, 15, 30, 60, 120, 240, 360, 480, 600, and 720 mg $L^{-1}$ of the active ingredient S-metolachlor were prepared from commercial products. Distilled water was used as a negative control, and aluminum was used as a positive control. Macroscopic analyses (germination and growth) were performed for the two species, and microscopic analyses (chromosomal and nuclear changes) were performed for *L. sativa*. Negative interference of the S-metolachlor-based herbicide on lettuce was observed for all macroscopic and microscopic parameters tested. In maize, there was no significant interference in germination; however, the herbicide interferes negatively in seedling development. In brief, the herbicide based on S-metolachlor has phytotoxic potential, just as discussed.

**Key-Word:** Fitotoxicity; Seedling development; Germination; Micronuclei; Citotoxicity; Genotoxicity.

## 1. INTRODUCTION

The use of pesticides is a common agricultural practice employed in a wide variety of crops [1]. Herbicides are one of the most used classes of pesticides worldwide, their aim is to control weed growth in agricultural fields [2]. Brazil figures as the country that most uses pesticides in the world and also as the country that most uses pesticides that are banned in other countries that are highly concerned with the environment [3]. The misuse of these pesticides negatively effects the environment [1].

Brazil is the third largest maize producer in the world [4], and the crop has the second largest cultivated area in the country [5]. Among the herbicides used in maize crops is S-metolachlor [6].

S-metolachlor is a member of the chloroacetamide herbicide family. It is commonly used as a selective pre- and post-emergence herbicide to control annual grasses and broadleaf weeds in different crops, such as maize, sunflower, soybean, sugarcane, cotton, beans, among others [6,7]. This herbicide acts by inhibiting various biological processes in the meristematic zones of plants. It is absorbed by the roots and aerial portions of germinating plants, and translocated via xylem to developing areas [8,9]. Once there it inhibits the synthesis of chlorophyll, proteins, fatty acids and lipids, preventing cell growth and division, and, consequently, blocking the growth of weeds soon after germination [8].

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The active ingredient S-metolachlor is relatively soluble in water and moderately absorbed by soil particles. When dissolved it can be transported in surface and underground water. It has the potential to contaminate underground water reserves due to its relatively long life in soil and water [10]. In this sense, studies that evaluate the effects of this environmental pollutant on living organisms are of great importance, as well as its toxicity mechanism considering how it acts on root and shoot growth and how it relates to changes at a cellular level.

Higher plants bioassays are efficient to identify the toxicity of environmental pollutants [11–14]. Macroscopic phytotoxicity tests are carried out through bioassays involving the germination of seeds of model species in addition to root and shoot growth of these model plants. Since the root is the first organ to come into contact with the substrate or solution containing the substance to be analyzed these tests are simple, fast, reliable and inexpensive [13]. Cytogenetic toxicity bioassays are tests that can be associated with macroscopic tests [15]. They are based on the assessment of the cell cycle, allowing the observation of events throughout mitosis [11,16], and can identify changes in the cell cycle by detecting abnormalities in the rate of cell division and also the presence of chromosomal and nuclear alterations [13].

Thus, this work aim was to evaluate the effects of an S-metolachlor-based herbicide on the initial development of plant models *Lactuca sativa* L. and *Zea mays* L. through macroscopic and microscopic bioassays.

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## 2. RESULTS

The S-metolachlor based herbicide affected the germination of lettuce seeds for all treatments, when compared to the negative control (water). This was more pronounced starting from the 120 mg $L^{-1}$ concentration, which is the dosage recommended for use by the manufacturer. At this concentration, more than 60% germination inhibition was observed (FIGURE 1). At the 360, 480 and 600 mg $L^{-1}$ concentrations a reduction of more than 90% in germination occurred, while the highest dose (720 mg $L^{-1}$) completely inhibited the germination of lettuce seeds.

It was also observed that the herbicide interfered with the germination speed of lettuce seeds: there was approximately a 50% reduction in the germination speed at the dosage range from the 7.5 to the 60 mg $L^{-1}$ treatments, 75% reduction for the 120 and 240 mg $L^{-1}$ treatments, and a 90% reduction at the highest concentrations (360, 480 and 600 mg $L^{-1}$) (Figure 1).

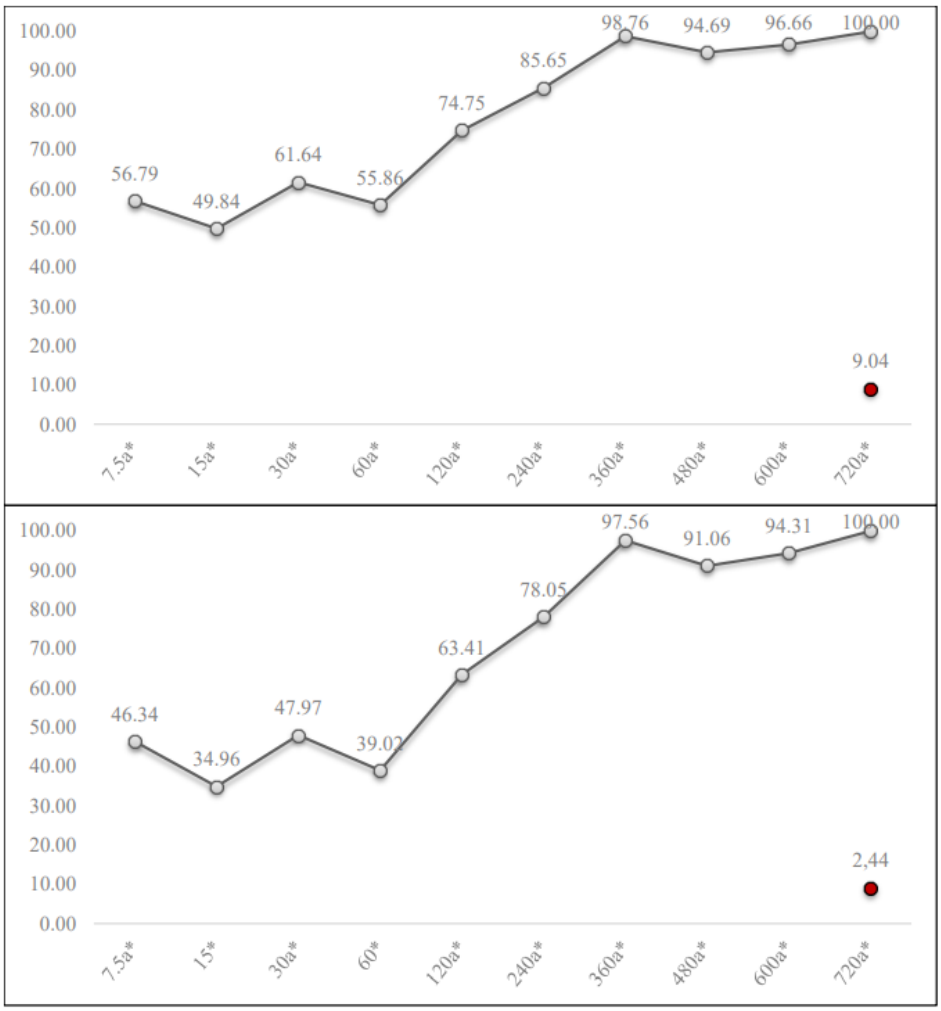


**Figure 1.** On the top: percentage of Germination Rate inhibition in relation to the negative control treatment (water) observed in *Lactuca sativa* seeds exposed to the S-metolachlor based solutions. On the bottom: percentage of Germination Speed Index (GSI) inhibition in relation to the negative control treatment (water) observed in *Lactuca sativa* seeds exposed to the S-metolachlor based solutions tested. The red dot represents the positive control (Al). Treatments followed by a "*" differ significantly from the negative control while values followed by the letter "a" differ significantly from the positive control.

For seedling development, which considered the measurements of the root and shoot, the herbicide had a negative impact for all concentrations tested, displaying more than 80% reduction for the lowest concentration. From the dosage of 120 mg $L^{-1}$ (recom-

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mended dosage for use) onwards there was more than 95% reduction in growth, and starting at the dosage of 360 mg $L^{-1}$ there was practically no seedling development at all (Figure 2). It is important to emphasize that every S-metolachlor treatment from that point on had a more pronounced effect than the aluminum, the latter was used as a positive control and is known to have great effect on stunting seedling development.

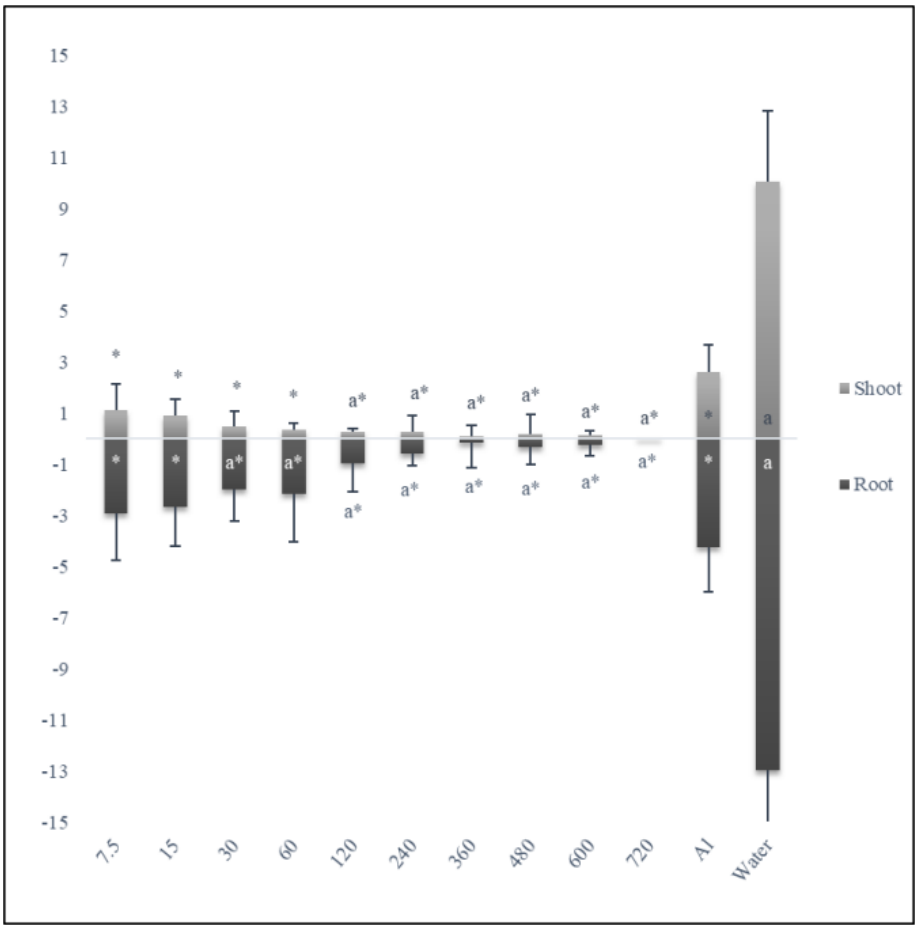


**Figure 2.** Root and shoot growth in millimeters observed in *Lactuca sativa* seeds exposed to the S-metolachlor based solutions tested. Bars are labeled with a "*" denoting significant difference from the negative control and/or with the letter "a" denoting significant difference from the positive control.

The cell cycle analyzes were performed in lettuce root tips for the treatments at the range from 7.5 to 60 mg $L^{-1}$, this was because on the other dosages it was not possible to obtain roots with adequate size for the preparation of slides due to severe root growth inhibition as discussed above. The herbicide containing S-metalochlor, when compared to the negative control, significantly decreased the mitotic index, by approximately 35%, for all treatments evaluated (Table 1).

As for the frequency of chromosomal alterations, the 60 mg $L^{-1}$ presented a significantly higher frequency than the negative control. The 7.5, 15.00 and 30 mg $L^{-1}$ treatments did not differ statistically from the negative control (Table 1). The chromosomal alterations found during this analysis were: c-metaphases, adherent chromosomes and fragments.

Regarding the frequency of micronuclei, the 30 $L^{-1}$ treatment differed from the negative control and the other treatments (7.5 15.0 and 60 $L^{-1}$) did not (Table 1). The 30 and 60 mg $L^{-1}$ treatments showed a higher frequency of condensed nuclei when compared to the negative control. The 7.5 and 15 mg $L^{-1}$ treatments did not differ statistically from the negative control for that end point (Table 1).

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**Table 1.** Cytogenetic analysis on root tips of *Lactuca sativa* exposed to solutions of S-metolachlor based herbicide.

| Concentrations (mg $L^{-1}$) | Mitotic Index | Chromosomal Alterations | Condensed Nuclei | Micronuclei |
|---|---|---|---|---|
| C- | 16.74a | 0.00a | 0.40a | 0.20a |
| C+ | 11.88* | 1.80* | 10.00* | 8.80* |
| 7,5 | 9.28* | 0.00a | 0.60a | 2.60a |
| 15,0 | 8.96* | 0.40a | 0.80a | 3,58a |
| 30.0 | 10.88* | 0.00a | 6.60* | 4.40a* |
| 60.0 | 9.38* | 0.80a* | 8.40* | 3,80a |

The Mitotic Index is in percentages. The values for Chromosomal Alterations, Condensed Nuclei and Micronuclei are in frequency per one thousand cells. Values followed by a "*" differ significantly from the negative control while values followed by the letter "a" differ significantly from the positive control.

Lastly, for maize, one of the cultures where S-metolachlor-based herbicides are applied commercially as a way to control weeds, no statistically significant reduction in germination was observed for any of the treatments when compared to the negative control (Figure 3).

On the other hand, the germination speed, when compared to the negative control, was negatively influenced, starting at the 360 mg $L^{-1}$ treatment (Figure 3) and proceeding. At the 720 mg $L^{-1}$ concentration there was more than 50% reduction in germination speed.

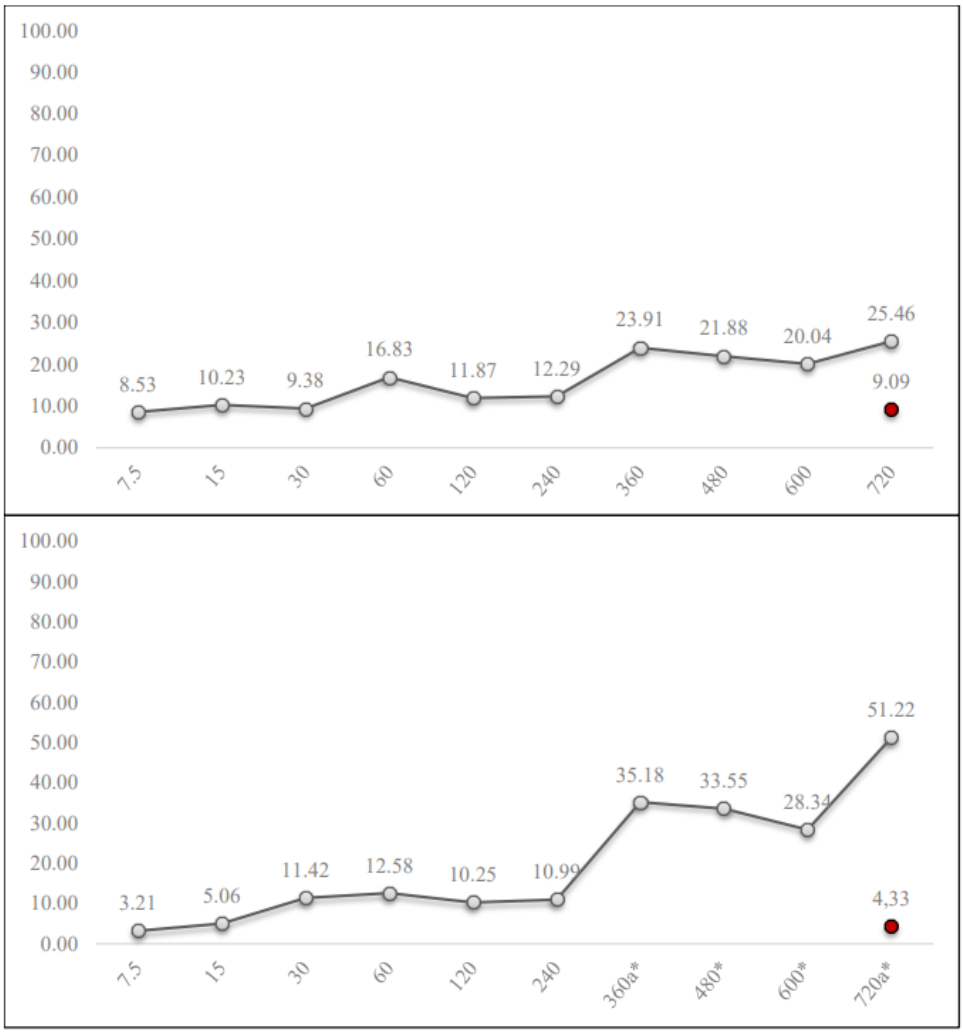

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**Figure 3.** On top: percentage of Germination Rate inhibition (%) in relation to the negative control treatment (water) observed in *Zea mays* seeds exposed to the S-metolachlor based solutions tested. On bottom: percentage of Germination Speed Index (GSI) inhibition in relation to the negative control treatment (water) observed in *Lactuca sativa* seeds exposed to the S-metolachlor based solutions tested. The red dot represents the positive control (Al). Treatments followed by a "*" differ significantly from the negative control while values followed by the letter "a" differ significantly from the positive control.

The S-metolachlor based herbicide affected the development of seedlings at all concentrations tested, but mainly at the highest concentrations (360 to 720 mg $L^{-1}$) (Figure 4). At the lowest concentration, 7.5 mg $L^{-1}$, there was a reduction of approximately 40% for root development and over 30% for shoot. At the dosage recommended for use by the manufacturer (120 mg $L^{-1}$) there was more than 60% reduction in root growth and approximately 50% in the aerial part and at the highest concentrations (360 and 720 mg $L^{-1}$) there was a reduction exceeding 80% in the development of the seedling as a whole (Figure 4).

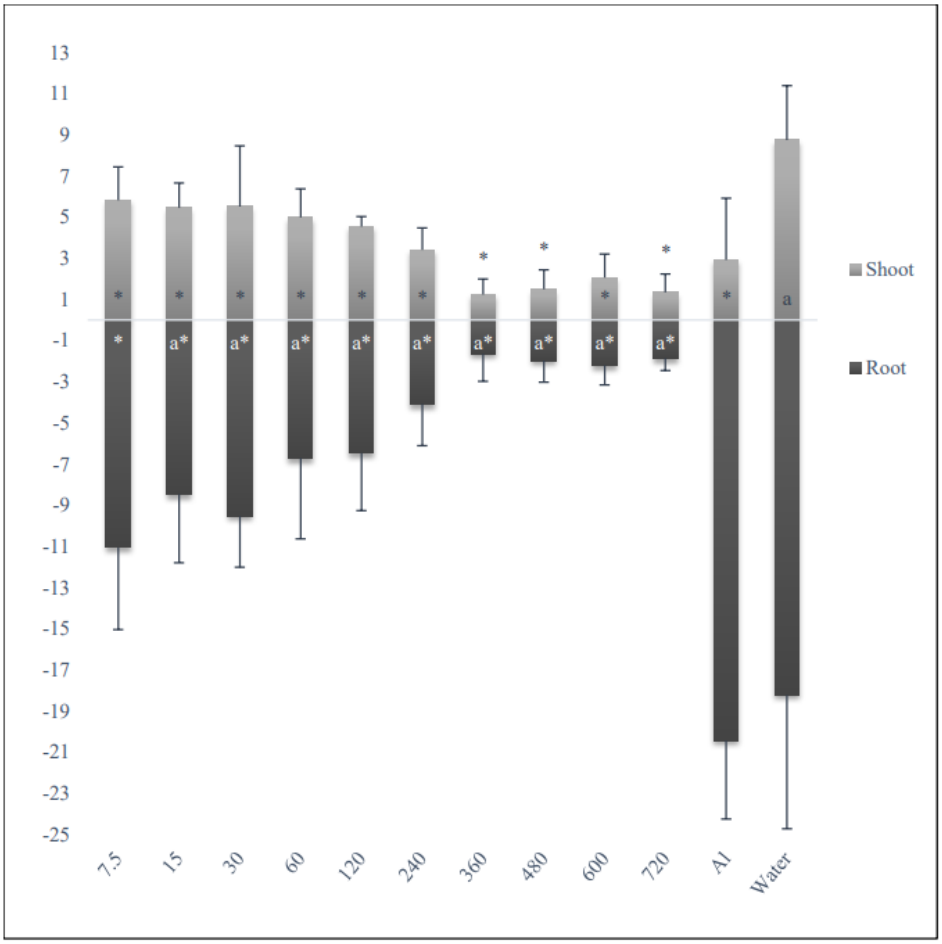


**Figure 4.** Root and shoot growth in millimeters observed in *Zea mays* seeds exposed to the Nicosulfuron based solutions tested. Bars are labeled with a "*" denoting significant difference from the negative control and/or with the letter "a" denoting significant difference from the positive control.

## 3. DISCUSSION

Evaluating the results obtained in this work as a whole it is possible to affirm that S-metolachlor-based herbicides do not prevent shoot or root growth, the seeds do germinate. However, the herbicide has an effect reducing the speed of germination and a con-

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sequence of it has a direct impact on seedling development presenting a phytotoxic effect, stunting plant growth mainly on the shoot.

Herbicides form the chloroacetamide family, like the S-metolachlor active ingredient, are known to be inhibitors of the root and apical meristems. According to Karam et al. [17] susceptible plants are stunted before emerging, without, however, completely stopping seed germination or immediately halting growth. These factors were observed in this study on both plant models. The authors also state that root growth is less affected than shoot growth which corroborates the observations made in this work.

Lettuce was more sensible to the effects of the herbicide than maize. On lettuce, as the dosage of the herbicide increased, a respective decreased in plant development was also observed. Maize, on the other hand, is not considered a target plant for this herbicide, S-metolachlor is used on maize crops and the plant is tolerant to it [17]. Even so the present study showed it was still affected by the herbicide even on dosages lower than the recommended for usage.

The selective of this herbicide is related to the metabolic rates of the exposed plant. Those that are more tolerant to it rapidly metabolizes it while the ones more subjective don't. Resistant plants like maize and soy (also not a target plant) are capable of metabolizing great quantities of the herbicide, enough to prevent its accumulation and persistence at phytotoxic levels. Absorption and translocation also appear to have a role in how much a plant is susceptible to S-metolachlor, the ones that are more vulnerable to it translocate the active ingredient to the growth portion of the plant following its absorption by the root [17].

This study marks the first time plant bioassays that evaluate root and shoot growth, using maize and lettuce as models, were used to evaluate the toxicity of an S-metolachlor based herbicide. For that reason, other works involving the same methods of exposition and application of test solutions in Petri dishes directly over the seeds that could be used as a basis for comparison weren't found in literature.

The effects of S-metolachlor on the root growth of maize (herbicide dosages of 6.2, 18.6,37.2, 74.4 and 93 μM) and rice (herbicide dosages of 0, 1.55, 3.1, 6.2, 12.4, 24.8 and 31 μM), both grown in hydroponic, were studied by Liu et al. [18]. There authors describe that the herbicide was toxic for both plants studied, reducing root growth and seedling development, data that is similar to the observed in this work. The authors also observed that maize was less sensible to the effects of S-metolachlor than rice, a fact that is also in accordance to what was observed here.

It was impossible to find in literature any papers evaluating the effects of S-metolachlor on lettuce. In works done using other eudicots as models the authors found the herbicide to be phytotoxic. The same is the case in the present study, where it was possible to observe S-metolachlor's toxicity having a detrimental effect on the development and cell cycle of *Lactuca sativa*.

In a field experiment lead by Sikkema et al. [19] using bean (*Phaseolus vulgaris*) as model (S-metolachlor applied on the dosages of 1373 e 2746 g/ha) was stated that the herbicide only causes minimal damage when applied on open field (chlorosis and necrosis of leaves, bent leaves and reduction on leaves growth rate). No adverse effect was observed on plant high, shoot dry weight, seed humidity and yield, which differs from what was observed here, where the active ingredient tested did provoke a reduction in plant growth.

Kalsing and Vidal [20] , in a study conducted in a greenhouse (Dual Gold® 915 g $L^{-1}$, using dosages of 1200, 1800, 2400 and 3600 g/ha) it was again shown that the herbicide ha dno significative effect on the emergency of bean plants, regardless of the dosage tested. However, there was an effect reducing the root dry mass and shoot dry mass (the later only for the highest concentration tested). A similar effect was observed here in this work, where S-metolachlor did not stop lettuce seeds from germinating but a reduction in the speed which that happened as well on the development of the root and specially the shoot was noticed.

 doi:10.20944/preprints202209.0351.v1

According to another field work done by Santos et al. [21], S-metolachlor (1.14 kg/ha) causes injuries, reduces plant growth and also the amount of dry matter on bean. On this same study, evaluating the effects of the herbicide on soy (*Glycine max*), an eudicot, the active ingredient reduced growth, dry matter e had moderate phytotoxicity. Once again these results are similar to those encountered by the present work when evaluating the effects of the herbicide on lettuce, also an eudicot. However differently from what was seen here, Santos et al. [21] found that S-metolachlor did not affect plant development when maize was exposed to the treatments.

An experiment using sweet potato (*Ipomoea batatas*), another eudicot, as model (S-metolachlor used in dosages of 0.00, 0.86, 1.72, 2.58 and 3.44 kg/ha) was conducted in a controlled environment by Abukari et al. [22]. The results found by the authors corroborate those found here in this study when evaluating lettuce. They observed that the herbicide caused a reduction on the total length of the plant, and this reduction increases as the dosage of the active ingredient does so as well. The authors also found that this was also true for other parameters, like number of roots, leaves, root and steam biomass and total area covered by leaves.

Santos et al. [23] during field researches (S-metolachlor used in a 1.14 kg/ha dosage) targeting weeds of the tomatoes (*Solanum lycopersicum*) crop, showed that the herbicide was more effective in stunting the growth of broad leave weeds (*Portulaca oleracea, Trianthema* spp., *Cleome viscosa*, *Boerhavia erecta, Amaranthus dubius*, *Echinochloa colona*, *Digitaria* spp. e *Echinochloa indica)* (eudicots), affecting the weeds density, than it was for narrow leave weeds (grass and *Cyperus*) (monocots) where a reduction in weeds density was also observed but it was less pronounced. This corresponds to what was observed in this current work, where lettuce, and eudicot, suffered greater effects of the active ingredient than maize, a monocot, did.

Gonçalves Netto et al. [24] , during an experiment in a greenhouse evaluating the effects of the herbicide (960 and 1440 g/ha) on the eudicot *Amaranthus palmeri* in two different types of soils, found S-metolachlor to be highly toxic to the plant, reducing its growth in over 90% and even causing them to die. The results observed on lettuce on this present study correlate to these findings.

The cytogenetic analysis were carried out for the 7.5, 15, 30 and 60 mg $L^{-1,}$ treatments, on the other dosages not enough material was obtainable for the preparation of slides. Like it was stated before, stating on the 120 mg $L^{-1}$ treatment onwards, a severe reduction on germination was observed and therefore collecting enough root tips for the analysis was not possible.

Root length is a parameter that relates with the rate of cell division and the mitotic index indicates this rate of division [25]. On the present work this relation was very evident, during the cytogenetic analysis it was observed that the herbicide interfered on the mitotic index and that was expected once you consider that the active ingredient tested also provoked a reduction on root and shoot growth as well as on the seedling as a whole. This happens because the increase in the number of cells is required for the development of any plant organ, and the mitotic index indicates the rate of cell division [25].

Agents that reduce mitotic index have a tendency to also increase the frequency of condensed nuclei [25,26]. This was observed in the current study where the presence of condensed nuclei was observed on the tested dosages of 30 and 60 mg $L^{-1}$. This nuclear alteration denotes cytotoxicity of the substance under evaluation, nuclear condensation is one of the first phases during plant programmed cell death, a defense mechanism of the organism that aims to impede the widespread dissemination of genetic damage caused by the toxic agent [26]. It is believed that plants can activate this defense when the damage caused are above a certain threshold and cannot be repaired, this causes an increase in the number of condensed nuclei [27]. The results found here, where condensed nuclei were observed even on the lower concentrations tested, indicates that the plant was unable to repair at least part of the genetic damage caused by S-metolachlor triggering programed cell death.

 doi:10.20944/preprints202209.0351.v1

Chromosomal alterations are also a common consequence of the toxic activity on a plant of a substance. The genetic instability and the DNA damage caused by the action of toxic substance can create a wide variety of chromosomal alterations [27]. The S-metolachlor based herbicide provoked a significative increase in the number of chromosomal alterations on the 60 mg $L^{-1}$ treatment. C-metaphasis, adherent chromosomes and bridges were some of the alterations found here.

The presence of micronuclei is also another endpoint to evaluate DNA damage. The cell has a set of mechanisms designed to repair DNA damage, however when these don't work properly or are overloaded, mitotic alterations can arise. These alterations can lead to the formation of micronuclei during cell division [27]. Micronuclei are related to aneugenic mechanisms (effects provoked by malfunction of the spindle or of the chromosome structure) and clastogenic (effects directly on the DNA structure), and therefore may be formed by entire chromosomes or fragments [25]. This way, the micronuclei observed for the 60 mg $L^{-1}$ treatment can be related to the chromosome alterations also found on this treatment, specially bridges, an alteration that is frequently associated with the formation of chromosomal fragments [15].

As this study is a pioneer when it comes to testing the cytogenetic effects of a S-metolachlor based herbicide on a cytogenetic level using lettuce as model not many information could be found on literature to compare with the results found here. However, in a research using algae (*Chlorella pyrenoidosa)* as model the herbicide was observed to reduce the algae division. As the concentrations tested increase greater was the inhibition [28]. The authors also found that the active ingredient reduced cell size, provoked rupture of the cell membrane e other alterations like the increase in the amount of starch grains and lipid droplets. Maronić et al. [29] also observed similar results, mainly reduction in cell division, when testing the herbicide using the algae *Parachlorella kessleri* as model. These data correlate to what was found on the present study, where the herbicide provoked a reduction in mitotic index and induced the formation of alteration in the cell cycle.

## 4. MATERIALS AND METHODS

### *4.1. Plant Materials*

Seed from non-target plant model *Lactuca sativa* (lettuce) "Great Lake" variety was purchased in commercial agriculture stores while seeds from crop plant *Zea mays* L. (maize), UFLA JM100 cultivar, were obtained in inbreed bank from Federal University of Lavras.

The experiment was set in Completely Randomized Design, consisting of five repetition per treatment, corresponding to a Petri dish (9 cm diameter). Both plant seeds were disposed in Petri dishes with filter paper moistened with test solutions. Three milliliters of each test solution were applied in Petri dishes containing 20 lettuce seeds and 5mL in dishes with 15 maize seeds. During the time of exposure the Petri dishes were kept in the dark in a germination chamber at 24◦C [30].

### *4.2. Treatment Solutions*

A commercial solution of S-metolachlor (Dual Gold®) containing 960,00 mg $L^{-1}$ (4% m/v) of the active compound was used to prepared the treatments solutions. The commercial product was diluted in distilled water to obtained the final test concentrations (7,5; 15, 30, 60, 120, 240, 360, 480, 600 e 720 mg $L^{-1}$) applied in the study. Note that the concentration of 120 mg $L^{-1}$ is the one recommended to maize fields according to the product manufacturers, the other solutions were all based around this one (dividing or multiplying it). Ultrapure water was used as negative control (NC) and aluminum ($10^{-3}$ M) (prepared from $KAl(SO_4)_2$) x 12 $H_2O$), which is known to have phytotoxic effects in plant growth, as positive control (PC).

### *4.3. Macroscopic Analysis*

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The following macroscopic parameters were evaluated according to Aragão et al. [31]: germination percentage (G%) comprising seeds with radicle protrusion, after 48 h of exposure; germination speed index (GSI) with intervals of 8h of each annotation from 0 to 48h of exposure to treatments; root growth (RG), and shoot growth (PG). The measurements were in mm, determined with a digital caliper after 96 h of exposure.

*4.4. Microscopic Analysis*

The cyto-geno-toxicity of the S-metolachlor based-herbicide was evaluated in *L. sativa* root tips cells exposed to the 7,5; 15, 30, and 60 mg $L^{-1}$ treatments. After 96h of exposure to the above solutions and the controls (negative and positive), roots emitted were collected and fixed in and ethanol: acetic acid solution (3:1, v/v) and stored at –20 °C for at least 24 h.

The slides were prepared by the squashing technique [32]: fixed roots were washed with distilled water, hydrolyzed in HCl 1mol $L^{-1}$ at 60 ◦C for 9 -10 min, stained with Schiff Reactive, in dark, for 1.5 h., and finally stained with acetic carmine 2%. Each slide was prepared using two treated meristems with five slides (one from each Petri dish, repetition, in a given treatment) being evaluated per treatment. One thousand cells were evaluated per slide and a total of 5,000 meristematic cells were observed per treatment.

The slides were evaluated under light microscope and a total magnitude of 400 X and different mitotic division phases as well as possible chromosomal and nuclear alterations were observed and recorded. Mitotic index (MI) – percentage of each mitosis phase, percentage of chromosomal alterations (CA%), the frequency of micronuclei (MN) and percentage of nuclear alterations (NA) were obtained according to Palmieri et al. [27] .

*4.5. Statistical Analysis*

Data were subjected to ANOVA of all analyzed parameters (MI, %CA and MN). The Tukey test at 5% significance level was used for the comparisons. The whole statistical analysis was done using the open-source statistical software "R" [33].

*4.6. CONCLUSIONS*

This work is the first in literature to test the phytotoxic effects of an S-metolachlor based herbicide on the plant models lettuce and maize using the macroscopic bioassays of germination and of root and shoot growth, while applying the test solutions directly over the root tips in Petri dish. It is also the first to do so for the cell cycle evaluation cytogenetic bioassay.

Considering that the herbicide affected the germination of lettuce seeds in all of the treatments evaluated and that also, at the highest dosage analyzed, prevented germination altogether, it was possible to conclude that herbicides based on S-metolachlor have a highly toxic potential for non-target plant species. The herbicide displayed moderately phytotoxic potential for maize as well, a crop for which it is actively used in agriculture, interfering in the seed germination speed. In addition, it affected both the root and the shoot growth of lettuce and maize. For lettuce at the highest dosage roots weren't even emitted and there was no shoot growth, this was observed starting from the concentration that is equivalent to the recommended usage dosage by the manufacturer. The herbicide also affected lettuce's mitotic index at all concentrations tested. Furthermore, S-metolachlor induced the formation of chromosomal alterations, micronuclei and condensed nuclei on the slides analyzed.

To summarize, the S-metolachlor-based herbicide was toxic to plant models, evidencing the risks for the environment its use can have. This danger is further exacerbated by the fact that, even on the lowest dosages tested, damage leading to death of the studied plants was observed. This underlines the dangers that S-metolachlor can present, especially if diffused in bodies of water. Considering that lettuce (eudicot) was more sensible to the toxicity than maize (monocot), it is possible to infer that the herbi-

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cide may be toxic to native eudicot plants close to the crops in which it was used, leading to an unbalanced selection of native species.

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**Author Contributions:** L. F. Andrade-Vieira concepted and design the work; Q. M. Silva organized the experiments, collected the data and drafting the article; M. J. Palmieri analyze and interpreted the data collected; L. F. Andrade-Vieira critical review the article and approved the final version to be published.

**Availability of data and material :** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Additional Information:** Larissa Fonseca Andrade Vieira is a researcher with ten years of experience in phy-geno-toxic studies and all the experimental design applied here was derived from wide experiments to test the best conditions to performed it.

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