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RESEARCH ARTICLE

Toxicity of paclobutrazol-based pesticide on *Lactuca sativa* L.: germination, seedling development, and DNA damage

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Abstract

Paclobutrazol, a fungicide of the triazole class, is widely used as an inducer of early flowering and fruiting by inhibiting gibberellin formation. However, biological assays using model organisms to evaluate their cytogenotoxic and mutagenic potential are still scarce. Therefore, this study aimed to investigate the effects of the commercial product Cultar® 250 SC (CP) and the pure substance (PBZ) on the germination and initial seedling development of *Lactuca sativa* L. (lettuce), in addition to evaluating the effects of CP on the mitotic activity and DNA, as we believe that PBZ has a greater toxic potential than CP on seed germination, and that the latter has cytogenotoxic and mutagenic effects on *L. sativa*. Lettuce seeds treated with CP and with PBZ in the doses of 0.25, 0.50, 1, 1.5, and 2 g L^{-1} showed significant reductions in germination rate, as well the CP reduced the root and initial development seedling development. PBZ showed greater inhibition of germination compared to CP. In direct exposure to PBZ, there was not sufficient seedling development for analysis, while in discontinuous treatment, there was inhibition of root growth (except for doses of 0.25 and 0.50 g L^{-1}) and in the development of the aerial part. While no mitodepressive effect was observed in meristematic cells treated with CP, increased frequencies of chromosomal alterations, including condensed nuclei and micronuclei, were evident in both meristematic cells and the F1 region. The Comet assay further demonstrated higher levels of DNA damage at higher paclobutrazol doses, supporting the findings of increased micronucleus frequencies. Consequently, it can be concluded that the CP exhibits greater toxicity towards seed germination compared to lettuce seedlings, and PBZ has a greater toxic potential than CP in relation to these parameters. However, the impact of CP on seedlings was relatively minimal, as evidenced by their limited effects on development, cell proliferation, and DNA, suggesting a slight toxicity of this agent. Therefore, we infer that Cultar® 250 SC should be used with caution. Thus, this study emphasizes the importance of employing joint analyses to better elucidate and correlate the mechanisms

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Highlights

- Paclobutrazol Inhibits Germination and Seedling Growth in Lettuce
- Chromosomal Alterations and DNA Damage Observed in Treated cells
- Cultar® 250 SC Impairs Germination but Minimally Affects Seedlings
- Importance of Varied Analysis Methods in Toxicity Assessment

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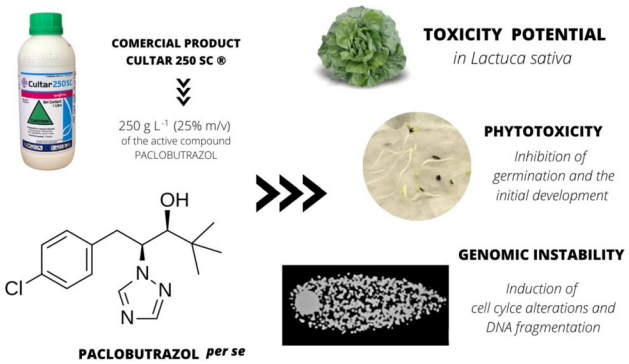
of action of potentially toxic substances. Furthermore, it provides a basis for discussing the application of Cultar® 250 SC and seeking more sustainable alternatives in food production.

Graphical abstract



Keywords Lettuce toxicity · Initial development · Micronuclei · Comet assay · Plant growth regulator

Introduction

Pesticides are essential in modern agriculture, significantly enhancing crop yield and quality, which is vital for global food security. Annually, about 2 million tonnes of pesticides are used worldwide to combat pests, as nearly 45% of food production is lost to pest infestation. However, while these chemicals boost productivity, they also accumulate in the environment—affecting soil, water, air, and the food chain—posing risks to human health and ecosystems (Sharma et al. 2019).

The Brazilian agricultural matrix is recognized for its remarkable diversity, making Brazil one of the most agriculturally diverse countries in the world. However, it is important to note that Brazil has also gained attention as the largest consumer of pesticides globally. When evaluating pesticide usage in relation to the cultivated area, Brazil ranks seventh. Similarly, considering the consumption rate per unit of agricultural product, Brazil ranks 13th (Carbonari and Velini 2021). These figures emphasize the significance of pesticide usage in Brazilian agriculture.

However, it is essential to further examine the implications of such consumption, particularly in terms of environmental impact concerning their genotoxic, mutagenic, and carcinogenic properties, even at low concentrations (Bolognesi and Morasso 2000; Fernandes et al. 2009, 2007). The risks associated with pesticides extend beyond potential poisoning from consuming food with pesticide residues after harvesting, processing, or preparation (Gerage et al. 2017). There is also a risk of poisoning through ingestion or exposure to surface water and groundwater contaminated by leaching processes (Pérez-Lucas et al. 2019).

One specific pesticide, paclobutrazol (PBZ) (chemical name: (2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pentan-3-ol) is classified as a fungicide and belongs to the triazole chemical group due to the presence of an aromatic ring (Wang et al. 2015). Its primary functions include promoting flowering and fruit setting, regulating plant growth, and providing protection against fungal diseases. The use of PBZ has proven beneficial in enhancing crop productivity and quality by optimizing plant development and preventing losses due to fungal infections (Wang et al. 2019). The mode of action of PBZ involves the disruption of the isoprenoid biosynthesis pathway through the inhibition of the kaurene oxidase enzyme, leading to the suppression of gibberellin biosynthesis (Bewley et al. 2013). Consequently, this alteration results in an increase in cytokinin levels and the accumulation of terpenoid precursors, which are redirected towards abscisic acid synthesis (Soumya et al. 2017). The disturbance of hormonal balance caused by the application of paclobutrazol in agricultural practices can have detrimental effects on plant development, potentially compromising their growth and physiological processes.

Although the broad range of applications highlights the importance of PBZ in agricultural practices and crop management strategies, its application on agricultural land can eventually lead to water system contamination through rainwater and runoff. The high mobility and stability of the molecule in the natural environment raise serious concerns, as PBZ has been detected in groundwater and soil at sites where it was applied (Wang et al. 2019). Moreover, multiple lines of evidence suggest that the misuse of PBZ results in environmental contamination and poses risks to the health of humans and animals (Shi et al. 2012).

Consequently, biological tests are crucial tools for prospecting the toxicity of PBZ and studying its mechanisms of action, as the molecular basis of its effects remains unclear (Baderna et al. 2011; Vieira and Silveira 2018; Wang et al. 2019).

Biological assays using plants as model organisms are widely employed for toxic risk assessments due to their ease of conduct, cost-effectiveness, and the possibility of extrapolating the results to other model organisms, including animals and human cells (Ma 1999; Reis et al. 2017). Furthermore, these assays do not require evaluation by ethics boards and many of them are already approved by the World Health Organization (WHO) and the United States Environmental Protection Agency (US-EPA) (Grant 1994, 1999).

Among the various biological models, *Lactuca sativa* L. from the Asteraceae family has been confirmed as an efficient species for evaluating cell cycle alterations in the context of environmental toxicology, alongside the widely employed *Allium* test (Silveira et al. 2017). *L. sativa* demonstrates high sensitivity, rapid germination, and easily handleable roots, facilitating the application of cytogenetic and molecular techniques (Andrade-Vieira et al. 2014; Palmieri et al. 2014; Silveira et al. 2017). It possesses medium to large chromosomes (ranging from 2.8 to 5.5 µm) with a reduced number (2n = 18), which aids in microscopic analysis (Matoba et al. 2007; Lima et al. 2019).

The assessment of the cell cycle in meristematic cells allows for the identification of clastogenic and aneugenic effects induced by potentially toxic substances (Fernandes et al. 2007; Leme and Marin-Morales 2009). Furthermore, the presence of micronuclei in F1 cells, located just above the meristematic region, can indicate the mutagenic effects of the tested substance (Palmieri et al. 2016). The Comet assay, in turn, enables the observation of DNA fragmentation (Silveira et al. 2017). The combination of these tests provides a comprehensive analysis of the cytogenotoxic and mutagenic potential of the substance under investigation.

Within this context, given the scarcity of reports on biological tests with model organisms to evaluate the cytogenotoxic and mutagenic potential of PBZ, our objective was to understand the effects of a commercial product based on PBZ, as well as the pure compound itself, on seed germination and early stages of seedling development in *L. sativa*. Additionally, we evaluated the effects of the commercial product containing PBZ on the cell cycle and DNA of *L. sativa* root tip cells. To address this, we hypothesize that the pure compound has a more significant impact on reducing seed germination and initial development of *L. sativa* seedlings compared to the commercial product and that the commercial product demonstrates cytogenotoxic and mutagenic effects on *L. sativa.*

Material and methods

The seeds of *Lactuca sativa* L. var. *grandes lagos* (lettuce), Asteraceae family, were purchased in the local trade, and presented germination rate close to 90%, with analysis date less than 180 days from the date of the experiments (information contained in the seed packaging—Topseed Garden).

The potentially toxic agent used to treat the seeds has the commercial name of Cultar® 250 SC (Syngenta Proteção de Cultivos LTDA) and, according to the technical label, it presents in its formulation 250 g L^{-1} (25.0% m v^{-1}) of paclobutrazol (chemical formula: $C_{15}H_{20}ClN_3O$, CAS 76738–62-0) and 835 g L^{-1} (83.5% m v^{-1}) of other ingredients not informed. Pure pacloputrazol from Sigma-Aldrich was also used.

Experimental design

The concentrations used in this study were determined according to information in the package insert of the commercial product Cultar® 250 SC, based on the concentration of the product to be applied to the cultivation of *Mangifera indica* L. (mango), family Anacardiaceae, where the recommended dose is 2.0 to 6.0 mL (0.5 to 1.5 g L^{-1} of paclobutrazol) per meter of diameter of the crown of the tree.

From this point on, every time the acronym CP is read, it should be understood as the commercial product (Cultar® 250 SC) containing paclobutrazol, and PBZ as the pure substance.

Lettuce seeds were treated with ultrapure water (negative control) and with CP (diluted in water) and PBZ (diluted according to Vieira et al. (2017)): at concentrations of 0.25 g L^{-1} (half the lowest recommended dose), 0.50; 1 and 1.5 g L^{-1} (recommended use doses) and 2 g L^{-1} (double the average recommended dose)).

Five 9 cm diameter polystyrene Petri dishes (repetitions) were used for each treatment, containing 30 lettuce seeds in each dish, which received 3 mL of the tested solutions.

The experiment was organized into four groups. In groups 1 to 3, the seeds were treated directly with paclobutrazol solutions; group 1 was treated with CP for 48 h, group 2 with PBZ solutions for 48 h, and group 3 with PBZ solutions for 120 h. For group 4, the seeds were exposed discontinuously to the solutions: initially, the seeds were treated with ultrapure water for 24 h to allow soaking, and then the emitted roots were exposed for 48 h to the PBZ solutions. During the exposure periods of seeds and roots, the Petri dishes were kept in a BOD (Biochemical Oxygen Demand) incubator at 24 °C in the dark.

Seed germination and seedling development

The germination percentage and the germination speed index (GSI) were calculated according to Maguire (1962). The Petri dishes of groups 1, 2 and 3 were inspected every 8 h and the number of germinated seeds was recorded in a spreadsheet.

After the exposure periods described for each group, the measurements of the roots and aerial part of the seedlings were obtained with the help of a digital pachymeter. For statistical calculation purposes, those seeds that did not germinate were not assigned any value in the growth spreadsheet.

After measuring the group 1 seedlings, they were fixed in a mixture of Ethyl Alcohol and Acetic Acid in a 3:1 ratio and stored in a freezer at -4 °C.

Cytogenetic analysis

The slides were prepared with the tips of the roots from group 1—exposed for 48 h to CP solutions -, through hydrolysis, exposure to Shiff's reagent and crushing, as described by Silveira et al. (2017). The meristematic region of the roots was used and another just above this, F1 region as described by Palmieri et al. (2016). For the meristematic region the mitotic index (MI) and the chromosomal alterations (CA) and nuclear alterations were analyzed. For the F1 region the presence of micronuclei (MN) and condensed nuclei (CN) were observed. In each slide around 1000 cells were counted for the meristematic region and 1000 cells for the F1 region.

A total of 60 slides (10 for each treatment, 2 per repetition) were prepared and analyzed under a light field microscope (Carl Zeiss, Axio Lab; A1) with a 40X objective and a total magnification of 400X. Representative images of the alterations found were obtained with the microcamera (AxioCam ERc 5 s) attached to the microscope, with total magnification of 1,000X, using the AxioVision software.

Comet assay

A group of 18 Petri dishes with seeds treated as described in group 1—exposed for 48 h to CP solutions—were submitted to the Comet assay. This assay was divided into 3 blocks, containing one Petri dish (repetition) of each treatment in each block, and 2 slides per plate were prepared. Thus, in the end, results from 3 repetitions of each treatment were obtained.

The slides were prepared following the protocol of Jovtchev et al. (2001), adapted by Silveira et al. (2017).

For each Petri dish a slide was evaluated under a fluorescence microscope (Nikon Eclipse E400), with emission at a wavelength of 520–560 nm, at 40X objective. Representative images of each treatment were obtained with a microcamera (Nikon Digital Sight DS-U3) coupled to the microscope with the aid of NIS-Elements BR 4.0 software.

The nucleoids were ranked, visually, in *scores* from 0 to 4 according to tail and head size (Reis et al. 2017), which made it possible to infer the level of DNA fragmentation caused by the treatments by calculating the percentage of damage and arbitrary unit (AU), following recommendations by Collins (2004).

Statistical analysis

The data obtained were submitted to analysis of variance and the means were compared by the Tukey test ($p < 0.05$). All analyses were performed in R software (R Development Core Team 2014).

Results and discussion

Seed germination and seedling development

The germination percentage (%G) and the germination speed index (GSI) of lettuce seeds treated with the commercial product (CP) (Group 1) exhibited a significant decrease compared to the negative control (ultrapure water) for all tested doses (Fig. 1a and b). Observing the number of lettuce seeds germinated within the first 24 h of the experiment (Fig. 1c), it became evident that the negative control group had nearly all seeds germinated (average of 27.8 seeds with radicle protrusion). In contrast, the plates treated with CP at doses ranging from 0.25 to 2 g L^{-1} exhibited only 2 to 6 germinated seeds. These results provide an explanation for the lower GSI in the CP treatments compared to the negative control.

Lettuce seeds treated with PBZ for 48 h only germinated at the dose of 0.25 g L^{-1} (1 to 4 germinated seeds per Petri dish) (Fig. 2a). However, seeds exposed for 120 h germinated at the doses of 0.25 g L^{-1} (4 germinated seeds in one Petri dish) and 1.5 g L^{-1} (1 germinated seed in one Petri dish) (Fig. 2d). Thus, PBZ treatments significantly inhibited seed germination (Tukey, $p < 0.05$). Although some doses did not result in any germination, it occurred in a small number of seeds and only after 32 and 72 h of exposure (Fig. 2c and f), highlighting a significantly lower GSI compared to the negative control (Tukey, $p < 0.05$) (Fig. 2b and e).

One of the well-known toxic effects of PBZ is its ability to block the biosynthesis of gibberellins (GAs) (Singh Narvariya and Singh 2018). By inhibiting the activity of kaurene oxidase (KO), a multifunctional enzyme of cytochrome P450 that catalyzes oxidation reactions in the generation of

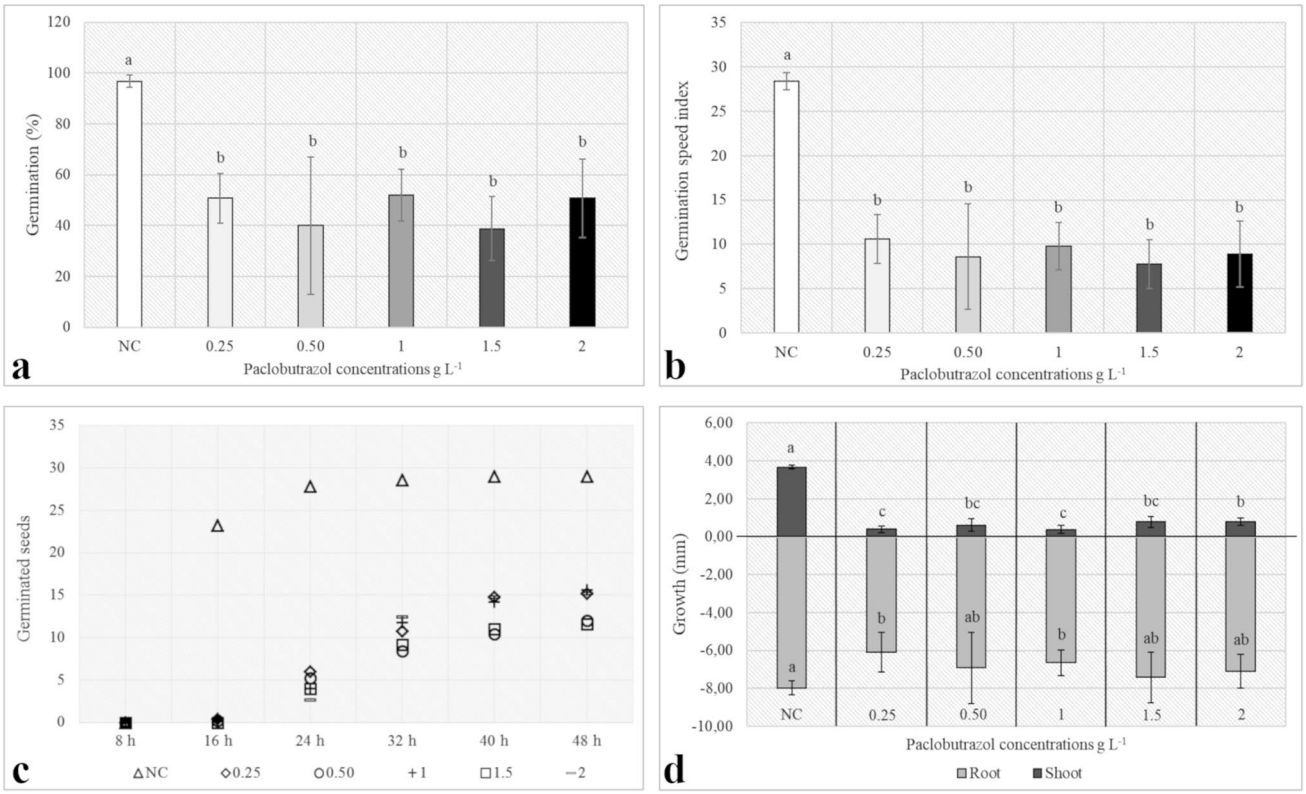


Fig. 1 Seed germination and initial development of *Lactuca sativa* L. (lettuce) seedlings treated with the commercial product Cultar® 250 SC, at concentrations of 0.25; 0.5; 1; 1.5 and 2 g L^{-1} of paclobutrazol. **a** Percentage of germination; **b** Germination Speed Index (GSI); **c** Number of germinated seeds; **d** Growth of roots and shoots

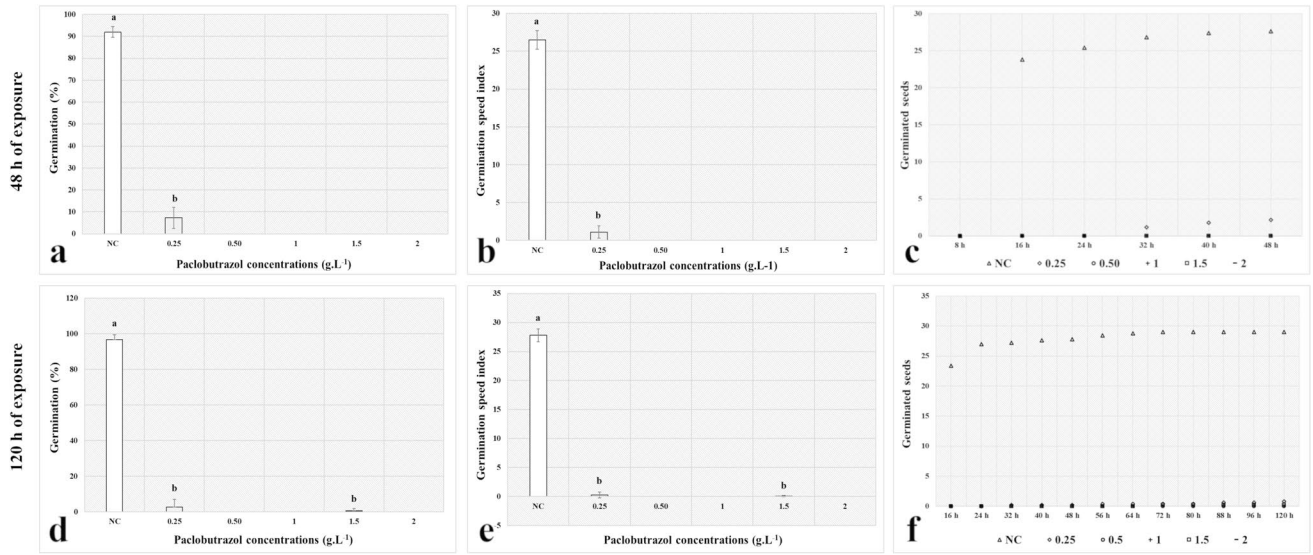


Fig. 2 Germination of *Lactuca sativa* L. (lettuce) seeds treated with paclobutrazol (pure substance), at concentrations of 0.25; 0.5; 1; 1.5 and 2 g L^{-1}. **a**; **b**; **c** *L. sativa* seeds treated for 48 h with paclobutrazol. **a** Percentage of germination; **b** Germination Speed Index (GSI); **c** Number of germinated seeds. **d**; **e**; **f** *L. sativa* seeds treated for 120 h with paclobutrazol. **d** Percentage of germination; **e** GSI; **f** Number of germinated seeds

bioactive gibberellin precursors, PBZ disrupts the biosynthesis process of this plant hormone (Morrone et al. 2010).

Gibberellins play a crucial role in activating the enzyme α-amylase, which, along with β-amylase, is responsible for the hydrolysis of reserves in the seed endosperm. These reserves are then transported to growth tissues and used in energy synthesis reactions essential for radicle development and emergence (Lopes et al. 2017). Therefore, the reduction in lettuce seed germination observed in the CP-treated group and the inhibition of germination in the PBZ-treated group can be attributed to the inhibition of gibberellin synthesis and subsequent non-activation of α-amylase and β-amylase during the second phase of germination. This effect may have been intensified by the pure molecule (PBZ treatments), emphasizing the necessity of de novo gibberellin synthesis for successful germination. On the other hand, the commercial product (CP) contains other constituents that might attenuate the primary effect of PBZ per se, resulting in only a delay in germination. Hence, the effect of the commercial product, at the concentrations used, may potentially be reversible.

After 48 h under the described experimental conditions, roots treated with doses of 0.25 and 1 g L^{-1} of CP exhibited the greatest reductions in growth (24 and 17%, respectively) compared to the negative control (Tukey, $p < 0.05$). The other doses (0.50, 1.5, and 2 g L^{-1}) showed reductions in root length in 14, 8, and 12%, respectively, but these reductions were not statistically significant when compared to the negative control (Tukey, $p < 0.05$) (Fig. 1d).

Regarding the aerial parts of the seedlings treated with CP, the control treatment exhibited the highest growth (3.67 mm). The doses of 0.25 and 1 g L^{-1} of CP resulted in the greatest reductions (90%), followed by 0.50 g L^{-1} (83%), 1.5 g L^{-1} (79%), and 2 g L^{-1} (78%). The results of all doses were statistically different from the negative control (Tukey, $p < 0.05$) (Fig. 1d). Although the treatment with 2 g L^{-1} was the highest dose of CP tested, it showed similar values to the control for root size (Tukey, $p < 0.05$) and exhibited the smallest reductions in the aerial parts (Fig. 1d). As previously mentioned, Cultar®, in addition to PBZ, contains other chemical compounds in its composition. Therefore, it is reasonable to infer that, since it is not a pure molecule, these other compounds may potentially interfere with the effect of PBZ on the initial development of seedlings.

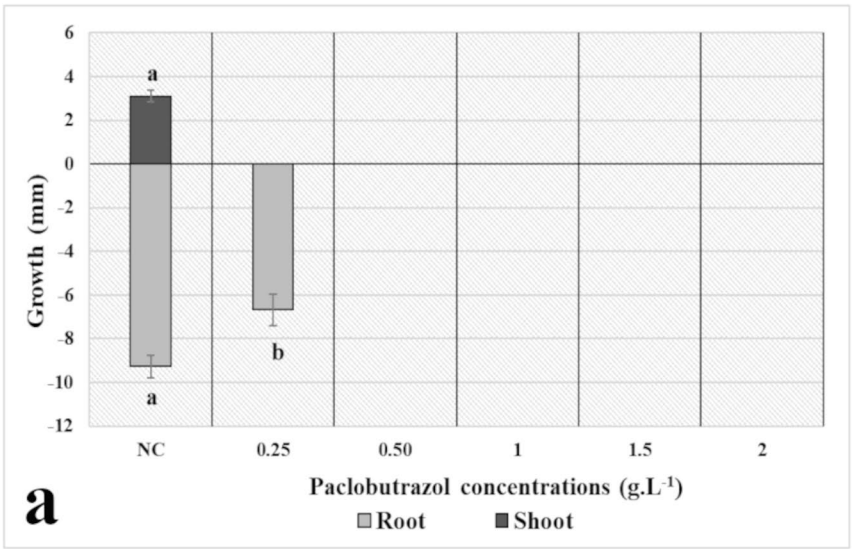
The roots produced by PBZ-treated seeds exhibited significantly less development compared to the negative control (Tukey, $p < 0.05$) in both the 48-h and 120-h exposure groups (Fig. 3a and b). Given the absence of observable seedling development across all doses during both exposure periods, a discontinuous treatment strategy was employed. In this approach, seeds were initially germinated in ultrapure water, followed by subsequent root exposure to PBZ for 48 h. In the context of this discontinuous treatment with PBZ, it was observed that roots treated with a dose of 2 g L^{-1} showed the greatest reductions in comparison to the control treatment. Moreover, in the aerial part, all doses significantly affected seedling development (Tukey, $p < 0.05$). Interestingly, the lower doses did not inhibit growth and even stimulated seedling development, as seen with the 0.25 g L^{-1} dose (Fig. 3c).

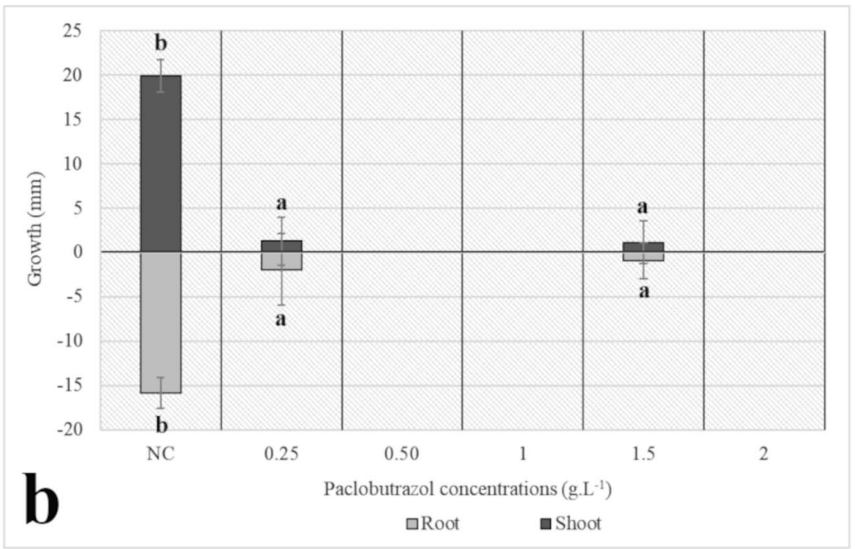
As previously discussed, PBZ directly affects the synthesis of gibberellins, thereby influencing radicle emergence. It is also known that alterations in the concentration of one hormone can trigger a cascade effect, influencing the synthesis of other hormones. In the case of seedling development, the plant hormone auxin plays a crucial role. Wang et al. (2015) reported that PBZ reduces the levels of auxin, specifically AIA (indole-3-acetic acid). Although hormone concentrations were not measured in the analyzed seedlings, it can be inferred that PBZ, both the pure compound and the commercial product, may have influenced a potential imbalance between these hormones, as indicated by the observed reduction in seed germination and initial seedling development in the CP and PBZ-treated samples.

In addition to its impact on seedling development, other authors have investigated the influence of PBZ on plant growth in various species. For instance, Manivannan et al. (2008) observed that PBZ increased root and stem length as well as leaf area of *Vigna unguiculata* (L.) Walp., particularly when combined with saline stress (100 mM NaCl). Similarly, in tomato plants (*Lycopersicon esculentum* Mill), Berova and Zlatev (2000) found that irrigation or foliar application of PBZ resulted in reduced plant height, increased stem thickness, accelerated root formation, and improvements in photosynthetic activity, water balance, and initial fruit production.

Chromosomal and nuclear alterations induced by Cultar®

It is known that during the germination process, there is accelerated cell division to facilitate rapid development of the embryo and protrusion of the root. Simultaneously, there is an investment by the seedling in the growth of its tissues, involving not only cell division but also cell elongation. Therefore, macroscopic observation of the initial development of seedlings can be associated with the mitotic index, as determined through microscopic analyses. In this context, we delved deeper into exploring the impact of PBZ in the CP on the cell cycle of lettuce seedlings. The analysis of lettuce root tip cells was exclusively conducted on seeds originating from the group 1 treatment, which were directly exposed to varying concentrations of the commercial product. While no significant differences were observed in the MI of CP-treated roots (Tukey, $p < 0.05$) (Table 1), the variations in growth among these roots could be attributed to factors beyond cell division, such as cell elongation. It is important to note that





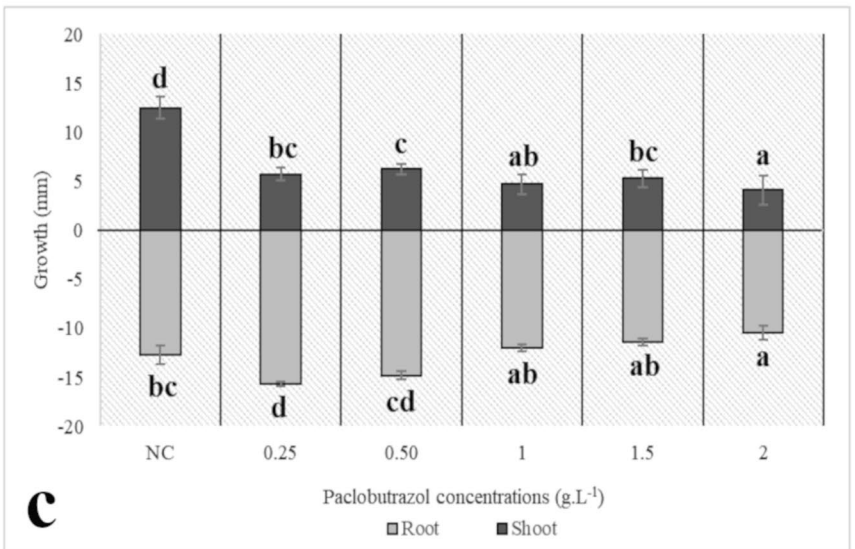


Fig. 3 Initial development of *Lactuca sativa* L. (lettuce) seedlings treated with paclobutrazol (pure substance), at concentrations of 0.25; 0.5; 1; 1.5 and 2 g L^{-1}. **a** Direct treatment for 48 h; **b** Direct treatment for 120 h; **c** Discontinuous treatment (after root protrusion) for 48 h

Table 1 Analysis of the cell cycle in meristematic cells of *Lactuca sativa* L. (lettuce) exposed to the commercial product Cultar® 250 SC, at concentrations of 0.25; 0.5; 1; 1.5 and 2 g L-*1* of paclobutrazol

Treatments	*N*	L	F	B	CM	S	TAC	TAD	MI
NC	0.00	0.00	0.00	0.00	0.00	0.06	0.06b ± 0.04	0.77b ± 0.55	7.60a ± 0.49
0.25 g L^{-1}	0.04	0.00	0.03	0.05	0.02	0.31	0.44a ± 0.17	5.98a ± 1.57	7.38a ± 0.64
0.50 g L^{-1}	0.02	0.01	0.00	0.04	0.00	0.25	0.32a ± 0.16	3.68ab ± 2.46	8.73a ± 0.89
1 g L^{-1}	0.05	0.02	0.04	0.09	0.02	0.20	0.42a ± 0.14	4.98a ± 1.41	8.37a ± 1.23
1.5 g L^{-1}	0.03	0.00	0.00	0.01	0.02	0.25	0.31a ± 0.10	4.22a ± 0.89	7.26a ± 0.38
2 g L^{-1}	0.03	0.01	0.02	0.07	0.02	0.28	0.43a ± 0.14	6.36a ± 1.27	6.73a ± 0.69

Legend: Mean followed by standard deviation. In the column, different letters mean that the results differed statistically according to the Tukey test ($p < 0.05$)

NC Negative control; *N* Unoriented chromosomes; *L* Lagging chromosomes; *F* Fragments; *B* Bridges; *CM* C-metaphase; *S* Stickiness; *TAC* Total alterations in total cells evaluated; *TAD* Total alterations in dividing cells; *MI* Mitotic index

root growth is a complex outcome influenced not only by cell division but also by the extent of cell elongation (Harashima and Schnittger 2010). The size of any plant organ ultimately hinges on the intricate interplay between these cellular processes.

Regarding the chromosomal alterations (CA) induced by the CP in meristematic cells (Fig. 4), observations included unoriented, delayed, fragmented, bridging, stickiness, and c-metaphase chromosomes. When considering the total frequency of these alterations, a significant difference was observed compared to the control treatment, but no difference was observed between the different concentrations of CP applied (Table 1) (Tukey, $p < 0.05$). When analyzing CA in relation to the number of dividing cells (Table 1), a low frequency of abnormalities was observed. However, the frequencies obtained from the treatments were significantly different from the negative control (Tukey, $p < 0.05$).

Among the CA observed in cells treated with CP, stickiness was the most frequent, with frequencies ranging from 0.20 to 0.31 (Table 1). The frequencies of other observed CA, such as unoriented chromosomes, lagging, fragments, and bridges, were less than 1% of the total observed cells (Table 1). However, these abnormalities showed an increase compared to the negative control, where they were not observed. The low frequency of these abnormalities can be considered within the range of spontaneous alterations that occur even in untreated cells.

It is worth highlighting, as emphasized by previous authors, that stickiness can be regarded as one of the most severe cell cycle alterations, given that the cell repair mechanism might struggle to reinstate the cell content to its normal state (Vieira and Silveira 2018). Hence, the most likely consequence of these stickiness is cell death. Stickiness is frequently linked with a condensed nucleus, often regarded as a cellular marker of cell death (Andrade-Vieira et al. 2011), alongside other concurrent nuclear modifications. In this context, the frequency of condensed nucleus in meristematic cells displayed a dose-dependent increase (Fig. 5 - a1, a2, and a3), reaching over 4% in treatments with 1.5 and 2 g L^{-1} doses. Although stickiness

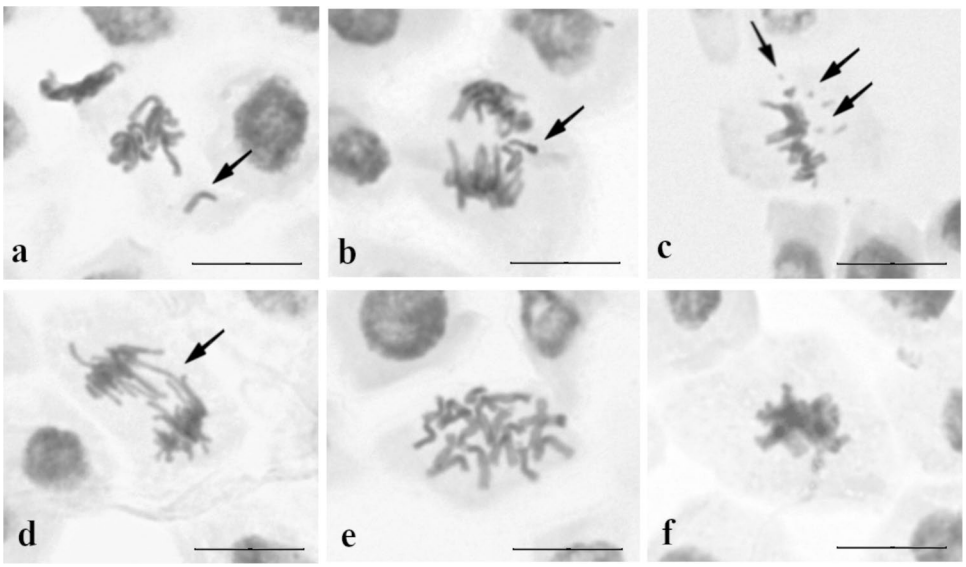


Fig. 4 Chromosomal alterations observed in meristematic cells of *Lactuca sativa* L. (lettuce) roots treated with the commercial product Cultar® 250 SC, at concentrations of 0.25; 0.5; 1; 1.5 and 2 g L^{-1} of paclobutrazol. **a** Unoriented chromosome (arrow); **b** Lagging chromosome (arrow); **c** Fragments (arrows); **d** Bridge; **e** C-metaphase; **f** Stickiness. Captures taken with a 100X objective. Bar = 10 µm

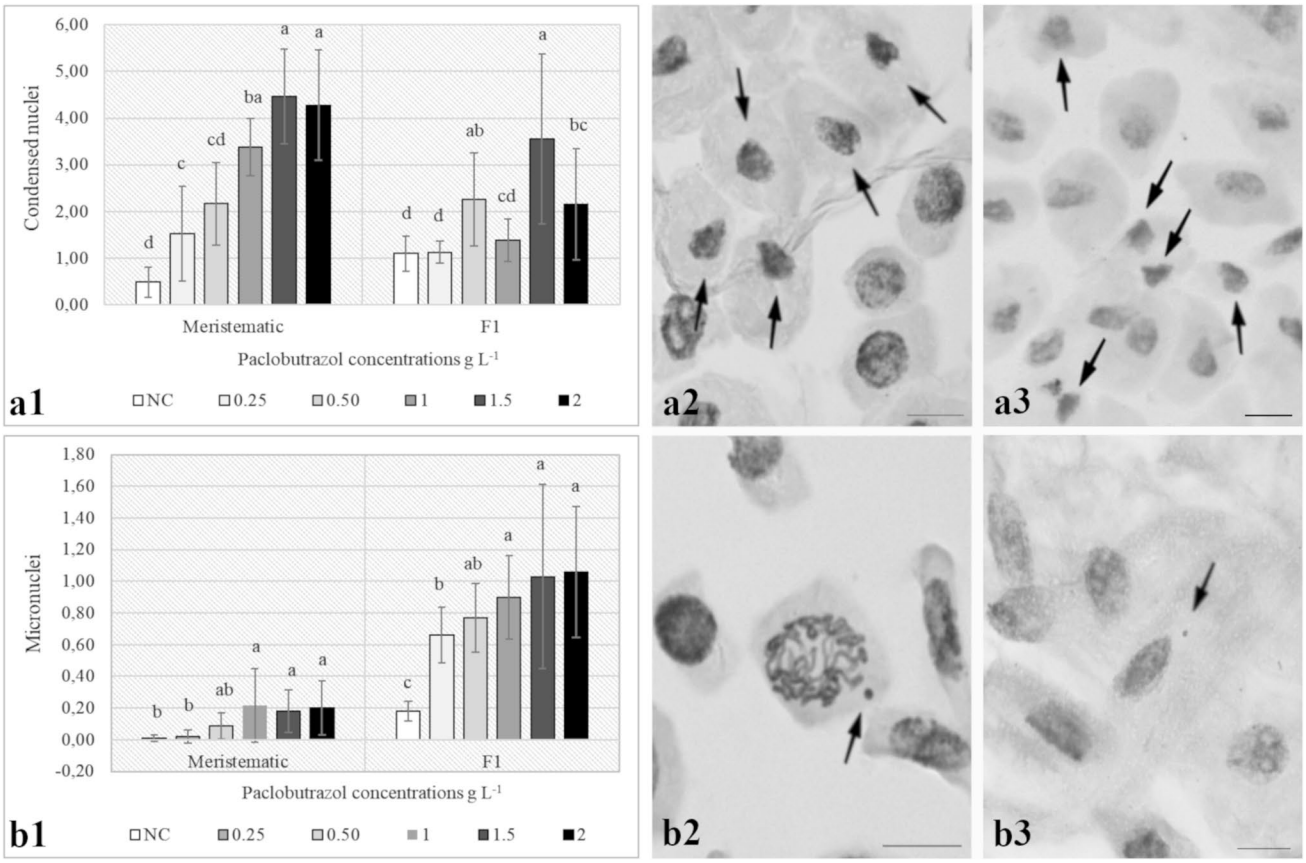


Fig. 5 Condensed nuclei and micronuclei observed in meristematic cells and the F1 region of *Lactuca sativa* L. (lettuce) roots treated with the commercial product Cultar® 250 SC, at concentrations of 0.25; 0.5; 1; 1.5 and 2 g L^{-1} of paclobutrazol. **a1** Frequency of condensed nuclei; **b1** Frequency of micronuclei; **a2** Nuclei condensed into meristematic cells; **a3** Condensed nuclei in cells in the F1 region; **b2** Micronuclei in meristematic cells; **b3** Micronuclei in cells in the F1 region. Captures taken with 40X (a3) and 100X (**a2**, **b2** and **b3**) objectives. Captures taken with a 100X objective. Bar = 10 µm

is linked with nuclear condensation occurrence, it is not the sole alteration triggering the cell death process. The incidence of condensed nuclei surpassed that of stickiness, implying that other factors and alterations also contribute to the activation of cell death.

Condensed nuclei also occurred in the F1 region (Fig. 5). In the roots treated with CP, the frequency of condensed nuclei ranged from 1.13 to 3.56, with the doses of 0.50, 1.5, and 2 g L^{-1} differing significantly from the negative control (Tukey, $p < 0.05$). The presence of condensed nuclei in this region suggests that although certain alterations do not impede normal cell division in the meristem, severe toxicity can activate the cell death mechanism when cells are in the process of differentiation. Evaluating F1 region is crucial to verify if the alterations caused by the treatments are being properly repaired since any breakage or fragmentation in the genetic material is expressed in the form of micronuclei in the subsequent cellular generation (Palmieri et al. 2016). Moreover, the combined evaluation of the meristematic region and F1, as reported by Palmieri et al. (2016), allows for comprehensive assessment of the main damages caused to the cells.

Considering in the analysis of CA within both the total evaluated cells and those undergoing division (Table 1), it is evident that although the cellular repair mechanisms did not rectify these alterations, they did not hinder the continuous cycle of cell division. Nevertheless, it is plausible that these alterations were passed on to subsequent generations of cells, possibly resulting in the formation of micronuclei as observed in Fig. 5 (b1, b2, and b3).

The frequency of micronuclei (MN) observed in meristematic cells were significantly different from the negative control (Tukey, $p < 0.05\%$) only in treatments with doses of 1, 1.5, and 2 g L^{-1} of CP (Fig. 5 - b1, b2, and b3). In the F1 region, located just above the meristematic region where cells begin the differentiation process, the occurrence of MN increased in a dose-dependent manner and significantly (Tukey, $p < 0.05$), with roots treated with doses of 1, 1.5, and 2 g L^{-1} showing the highest frequencies (0.9, 1.03, and 1.06, respectively) (Fig. 5 - b1, b2, and b3).

The occurrence of micronuclei (MN) in both meristematic cells and the F1 region can be attributed to various factors, including amplification and elimination of genetic material from the nucleus, chromosomal breaks, and loss of entire chromosomes during cell division (Fernandes et al. 2007; Fenech et al. 2011). Therefore, the presence of unoriented and delayed chromosomes, chromosomal fragments, and c-metaphases observed in meristematic cells aligns with the occurrence of micronuclei in both meristematic cells and the F1 region.

DNA damage induced by Cultar®

The observed micronucleus values in the meristematic region and F1 can be correlated with the percentages of DNA damage and the arbitrary unit (AU) values obtained from the comet assay since this test utilizes the root tip, encompassing both regions in question. The comet assay performed on CP-treated root tips revealed statistically significant differences from the negative control (Tukey, $p < 0.05$) in both the percentage of DNA damage and AU (Fig. 6).

In this study, AU values were preferred as they consider the severity of each damage, i.e., the score assigned to the nucleoids. The highest AU values were found in roots treated with the highest concentrations of CP (1.5 and 2 g L^{-1}, Tukey, $p < 0.05$) (Fig. 6). Roots treated with the other doses (0.25, 0.5, and 1 g L^{-1}) also showed significantly different values from the negative control (Tukey, $p < 0.05$).

Considering that AU values can range from 0 to 400, as described by Collins (2004), where 0 represents no damage and 400 represents the highest level of DNA damage, the observed AU values in this study indicate mild damage caused by CP to the lettuce DNA. This is confirmed by observing Fig. 6, which shows a large number of nucleoids classified as scores 1 and 2 in the treatments (averages between 11.67 and 51.33), and very few in scores 3 and 4 (averages between 0.33 and 9.67).

The micronucleus values obtained, both in meristematic cells and in F1, align with the AU values, where the last three doses exhibited the highest values for these parameters (Figs. 5 and 6). This further confirms the value of combining the comet assay with classical cytogenetic analyses to validate the observed abnormalities (Silveira et al. 2017).

Therefore, despite the slight nature of these damages, they represent direct alterations in the DNA molecule of *L. sativa*, predominantly observed at the highest doses tested. This suggests the necessity for cautious use of CP (Cultar® 250 SC) and underscores the importance of conducting further studies utilizing alternative techniques to

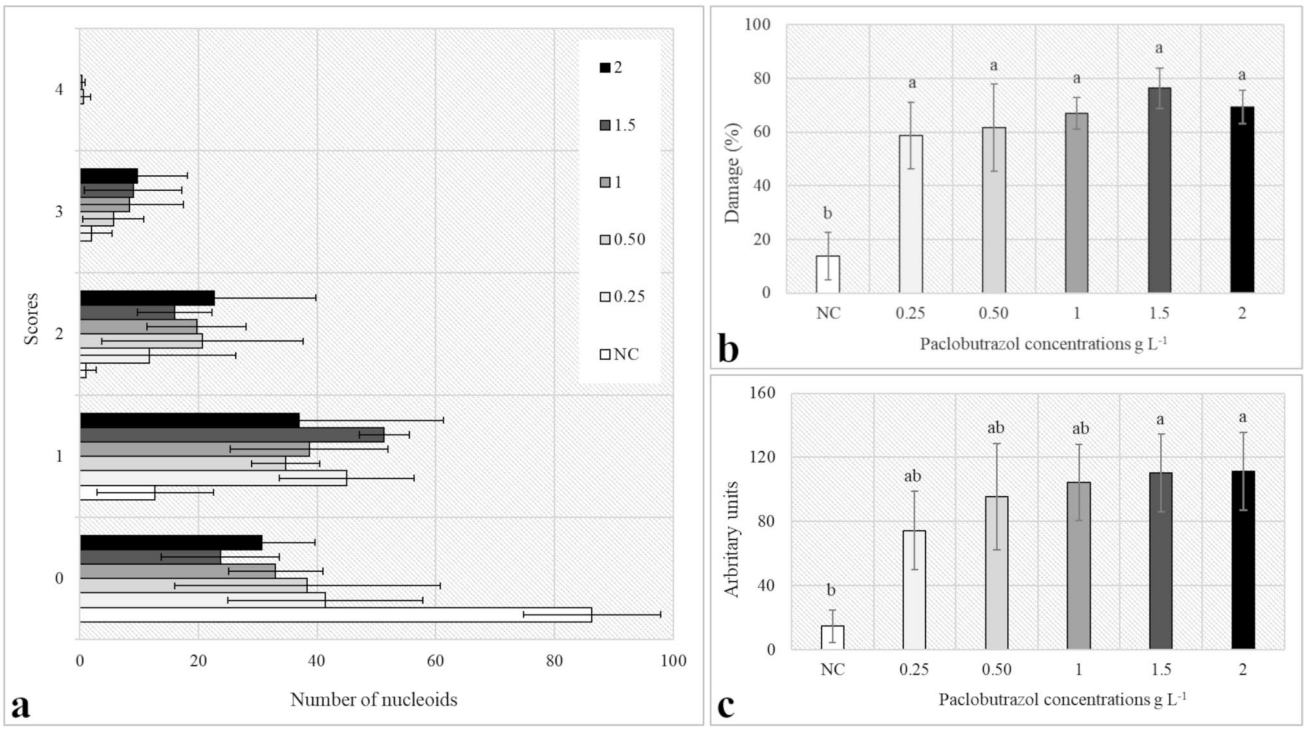


Fig. 6 DNA damage observed in root tip cells of *Lactuca sativa* L. (lettuce) treated with the commercial product Cultar® 250 SC, at concentrations of 0.25; 0.5; 1; 1.5 and 2 g L^{-1} of paclobutrazol. **a** Number of nucleoids observed in each score (0 to 4); **b** Percentage of DNA damage; **c** Arbitrary unit

assess the product's toxicity, thus enhancing our understanding of paclobutrazol.

Conclusion

The treatments with the commercial product (CP) containg paclobutrazol and with paclobutrazol per se (PBZ) caused reductions in germination and in root and initial development of *Lactuca sativa* L. (lettuce) seedlings. However, PBZ showed greater inhibition of germination compared to CP.

No mitodepressive effect was observed in lettuce meristematic cells, however, CP treatments increased the frequency of chromosomal alterations (CA), condensed nuclei, and micronuclei (MN). They also induced an increase in the frequency of condensed nuclei and MN in cells of the F1 region.

Although CA, condensed nuclei, and micronuclei were found in cells of the meristematic region of lettuce roots, it is possible to conclude that the treatments with CP caused mild toxic effect, which can be proven by the absence of mitodepressive effect and also by the low reduction in seedling development. Germination, on the other hand, was significantly affected by the treatments, suggesting that the seedling somehow managed to overcome and/or tolerate the toxicity of paclobutrazol and continue its development.

The AU values correlated with the micronuclei observed in the meristematic region and F1 showed that the highest doses caused the greatest damage, but that even so, this was slight when taking into account the number of micronuclei, as well as *scores* 3 and 4 nuclei observed.

Therefore, this study showed that the pesticide Cultar® 250 SC, which has in its formulation 250 g L^{-1} (25.0% m v^{-1}) of paclobutrazol, presents greater toxicity to the germination of lettuce seeds than to the seedlings, which were less affected in their development, cell proliferation and DNA, it can be said that this agent was slightly toxic.

Furthermore, the present work confirmed once again that joint analyses (seed germination, seedling growth, classical cytogenetics and Comet assay) are valid to better explain and correlate mechanisms of action of potentially toxic substances.

Thus, helping in the understanding of the mechanisms of action of this product, providing subsidies for discussions about its application in plantations and in the search for more sustainable alternatives in food production.

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Author contributions Author statement: Graciele Lurdes Silveira, Maria Gabriela Franco de Lima and Fábio Eduardo dos Santos: Investigation, Methodology, Data curation. Graciele Lurdes Silveira: Writing- Original draft, Writing- Reviewing and Editing, Visualization. Ingrid Fernanda Santana Alvarenga: Statistical Analysis. Elisa Monteze Bicalho: Reviewing and Editing, Conceptualization, Validation. Larissa Fonseca Andrade-Vieira: Writing- Reviewing and Editing, Conceptualization, Validation, Supervision, Project administration. All authors read and approved the final manuscript.

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Data availability The authors declare that the data supporting the findings of this study are available within the paper. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent to publish Not applicable.

Competing interests The authors declare no competing interests.

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