USE OF THE *ALLIUM CEPA* BIOASSAY AS AN INDICATOR OF CYTOTOXICITY OF PESTICIDES IN VITICULTURE

UTILIZAÇÃO DO BIOENSAIO *ALLIUM CEPA* COMO INDICADOR DE CITOTOXICIDADE DE PRAGUICIDAS DA VITICULTURA

UTILIZACIÓN DEL BIOENSAYO DE *ALLIUM CEPA* COMO INDICADOR DE CITOTOXICIDAD DE PLAGUICIDAS EN LA VITICULTURA

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Renata Sano Lini[1], Eduardo Daniel Righi Dornelles[2], Camilla Yumi Endo[3], Débora Hayane Vidal Pereira[4], Larissa Tiemi Akamine Motomura[5], Mariana Yumi Date[6], Thalita Fontana Manocchi[7], Priscilla de Laet Santana[8], Tânia Cristina Alexandrino Becker[9], Edilson Nobuyoshi Kaneshima[10], Simone Aparecida Galerani Mossini[11], Alice Maria de Souza Kaneshima[12]

ABSTRACT

Objectives: To evaluate the cytotoxic effect of pesticides used in grape cultivation in Marialva-Paraná through the Allium cepa bioassay.

Methods: Different concentrations of pesticides were tested: imidacloprid, Metiram+Pyraclostrobin, Metalaxyl-m+Mancozeb and Tetraconazole. The test solutions were prepared based on the recommended doses for grape cultivation and also with variations in these concentrations. Cytotoxicity was assessed by calculating the mitotic index.

[1] Dr. Universidade Estadual de Maringá. E-mail: renatalini23@gmail.com
Orcid: https://orcid.org/0000-0002-6161-2225 Lattes: http://lattes.cnpq.br/7572289351337886.
[2] Graduate. UNICESUMAR. E-mail: edu1.righi@outlook.com Lattes: http://lattes.cnpq.br/9966424013303468
[3] Graduate. Universidade Estadual de Maringá. E-mail: endocamillaa@gmail.com
Lattes: http://lattes.cnpq.br/6449211825495668.
[4] Graduate. Universidade Estadual de Maringá. E-mail: ra99624@uem.br
Orcid: https://orcid.org/0009-0004-5463-3089 Lattes: http://lattes.cnpq.br/2635068835023551
[5] Graduate. Universidade Estadual de Maringá. E-mail: larissamotomura@gmail.com
Lattes: http://lattes.cnpq.br/6600036124148799.
[6] Graduate. Universidade Estadual de Maringá. E-mail: ra117893@uem.br
Lattes: http://lattes.cnpq.br/0467454352429179.
[7] Graduate. Universidade Estadual de Maringá. Orcid: https://orcid.org/0009-0009-6719-9337
Lattes: http://lattes.cnpq.br/3254336568770278.
[8] Dr. Universidade Estadual de Maringá. E-mail: plsantana@uem.br
Orcid: https://orcid.org/0000-0001-6462-9631 Lattes: http://lattes.cnpq.br/0588488743824962
[9] Dr. Universidade Estadual de Maringá. E-mail: tcabecker@uem.br
Orcid: https://orcid.org/0000-0001-5952-6945 Lattes: http://lattes.cnpq.br/1536135079919278
[10] Dr. Universidade Estadual de Maringá. E-mail: enkaneshima@uem.br
Orcid: https://orcid.org/0000-0002-2725-8007 Lattes: http://lattes.cnpq.br/7618525126292006
[11] Dr. Universidade Estadual de Maringá. E-mail: sagmossini@uem.br
Orcid: https://orcid.org/0000-0001-9535-0983 Lattes: http://lattes.cnpq.br/9271272898352328
[12] Dr. Universidade Estadual de Maringá. E-mail: amskaneshima@uem.br
Orcid: https://orcid.org/0000-0002-9460-4643 Lattes: http://lattes.cnpq.br/2642062503039424

Results: Macroscopic analysis showed that, with increasing concentration tested, there was an increase in fragility and a decrease in root size. For all substances tested, there was a significant reduction in the size of the roots compared to the substances with the respective negative controls. Cytotoxicity was confirmed by calculating the mitotic index. Furthermore, during the counting of cells that presented different phases of cell division, some abnormalities were observed in the genetic material indicative of genotoxicity.

Conclusions: This study confirmed the cytotoxicity of all active ingredients tested, and the information presented aims to contribute to the development of additional research to relate cytogenotoxicity in humans.

Keywords: Mitotic Index. Biological Assay. Onions. Agrochemical.

RESUMO
Objetivo: Avaliar os efeitos citotóxicos de praguicidas usados no cultivo da uva em Marialva-Paraná por meio do bioensaio com Allium cepa.

Método: Foram testadas diferentes concentrações dos praguicidas: imidacloprido, Metiram+Piraclostrobina, Metalaxil-m+Mancozebe e Tetraconazol. As soluções-teste foram preparadas com base nas doses recomendadas para cultura da uva e também com variações nestas concentrações. A citotoxicidade foi avaliada por meio do cálculo do índice mitótico.

Resultados: Análise macroscópica mostrou que, com o aumento da concentração testada houve aumento da fragilidade e diminuição no tamanho das raízes. E para todos os ativos testados, houve redução significativa entre o tamanho das raízes em comparação dos ativos com os respectivos controles negativos. A citotoxicidade foi confirmada pelo cálculo do índice mitótico. Ademais, durante a contagem das células que apresentavam diferentes fases da divisão celular, foram observadas algumas anormalidades no material genético indicativas de genotoxicidade.

Conclusão: Este estudo confirmou a citotoxicidade de todos os ativos testados e as informações apresentadas visam contribuir para o desenvolvimento de pesquisas adicionais para relacionar a citogenotoxicidade em seres humanos.

Palavras-chave: Índice Mitótico. Bioensaio. Cebolas. Agroquímicos.

RESUMEN
Objetivo: Evaluar los efectos citotóxicos de plaguicidas utilizados en el cultivo de uva en Marialva, Paraná, mediante el bioensayo con Allium cepa.

Método: Se probaron diferentes concentraciones de los plaguicidas imidacloprid, Metiram + Piraclostrobina, Metalaxil-M + Mancozeb y Tetraconazol. Las soluciones de prueba se prepararon con base en las dosis recomendadas para el cultivo de la uva, así como con variaciones de dichas concentraciones. La citotoxicidad se evaluó mediante el cálculo del índice mitótico.

Resultados: El análisis macroscópico mostró que, con el aumento de la concentración, hubo mayor fragilidad y disminución del tamaño de las raíces. Para todos los principios activos evaluados, se observó una reducción significativa en el tamaño de las raíces en comparación con sus respectivos controles negativos. La citotoxicidad fue confirmada mediante el cálculo del índice mitótico. Además, durante el conteo de las células en

diferentes fases de la división celular, se observaron algunas anomalías en el material genético indicativas de genotoxicidad.

Conclusión: Este estudio confirmó la citotoxicidad de todos los principios activos evaluados, y los hallazgos buscan contribuir al desarrollo de futuras investigaciones para relacionar la citogenotoxicidad en seres humanos.

Palabras clave: Índice Mitótico. Bioensayo. Cebollas. Agroquímicos.

1 INTRODUCTION

Pesticides are widely used to combat pests and diseases to increase agricultural productivity. According to the annual bulletin of the Brazilian Institute of the Environment and Renewable Natural Resources (1), in 2022 more than 800 tons of formulated products were sold in the country.

The System for the Control of Trade and Use of Pesticides in the State of Paraná (SIAGRO) receives the sales declarations of all companies in the State, and in 2023, it registered more than 138 thousand tons of assets sold in Paraná. The municipality of Marialva was responsible for the sales of 544.6 tons of pesticides in the same year (2). In the last 3 years (2021, 2022 and 2023), pesticide sales have increased by more than 70 thousand tons (2), demonstrating that this sector is growing.

The update of the toxicological classification of pesticides by the National Health Surveillance Agency (3) added to the transformations in agricultural processes, indicate that the consumption of pesticides in Brazil should be intensified in the coming years. Insufficient occupational protection and monitoring measures in the area of agriculture increase and aggravate the public health problems caused by the wide exposure of the population and rural workers to pesticides. In particular, family farmers who are in a situation of social vulnerability (4).

Bioassays using higher plants are excellent indicators of cytotoxicity and genotoxicity of chemical substances, being used mainly in the detection of environmental pollutants (5). *Allium cepa*, commonly known as onion, is one of the species widely used in bioassays due to its ease of access, low cost, and low complexity in cultivation (6, 7). This species is indicated to evaluate several genetic parameters, having 16 large chromosomes, which facilitates the visualization of chromosomal abnormalities. In addition, the analysis of the cell cycle of *Allium cepa* roots allows the cytotoxic activity of different substances to be determined by evaluating the decrease in the frequency of cell division (8).

In addition, the bioassay with *Allium cepa* has great sensitivity in the evaluation of cellular toxicity and chromosomal alterations, contributing to the establishment of direct and/or indirect risks in the human population (9). Thus, the objective of this work was to evaluate the cytotoxic effects of pesticides used in grape cultivation in the region of Marialva in Paraná.

2 MATERIAL AND METHODS

2.1 BIOASSAY WITH *ALLIUM CEPA*

The onions (*Allium cepa*) used for the test were purchased commercially at a street market in Maringá-PR, from the same supplier. Bulbs with a diameter of approximately 3 centimeters were selected. All onions were sanitized with running water and the surface skins were removed.

2.2 TEST SOLUTIONS

All the pesticides tested were donated by a local producer, from Marialva-PR, who removed from the commercial packaging, the amount necessary to carry out the bioassay. Table 1 shows the trade name, active ingredient, classification as to purpose and toxicity of the pesticides tested. The information was obtained from the database of the Agricultural Defense Agency of Paraná (10).

Table 1

List of pesticides used for the Allium cepa bioassay

Trade name	Active	Class	Toxicological classification
Cabrio Top®	Metiram + Pyraclostrobin	Fungicide	4 - Slightly toxic
Proved 200 SC®	Imidacloprid	Insecticide	4 - Slightly toxic
Ridomil Gold- MZ®	Metaxyl-m+mancozeb	Fungicide	5 - Unlikely to Cause Acute Damage
Domark 100 EC®	Tetraconazole	Fungicide	4 - Slightly toxic

The pesticides tested are used during grape cultivation in the North Central region of Paraná. Therefore, the concentrations selected for this study were based on the doses recommended for grape cultivation, and concentrations lower and higher than those used for some cases were also used. Concentrations with cytotoxic and/or genotoxic effects described in the literature were also selected, as shown in Table 2. The lower concentrations were used to verify if, at these concentrations, the active ingredient still presented cytotoxic effects. The higher concentrations were used as a positive control, and it was expected to find a cytotoxic effect. All test solutions were prepared in a properly calibrated volumetric flask, taking into account the guidelines described in the package insert for the preparation of the mixture.

Table 2

Concentrations of each active ingredient used for the Allium cepa bioassay

Active	Concentration	Reason for the choice
Metiram + Pyraclostrobin	4g/L	Concentration of use for grape cultivation
	8g/L	Concentration 2 times higher than that of use

	16g/L	Concentration 4 times higher than that of use
Imidacloprid	0.2ml/L	Concentration of use for grape cultivation
	0.25ml/L	Concentration of use for grape cultivation
	0.40ml/L	Concentration of use for grape cultivation
	0.50ml/L	Concentration of use for grape cultivation
	8.75 ml/L	Concentration described in the article by Fioresi et al. (27) as potentially genotoxic
Metaxyl-m+mancozeb	6.25g/L	Concentration 4 times lower than that of use
	12.5g/L	Concentration 2 times lower than that of use
	25g/L	Concentration of use for grape cultivation
	50g/L	Concentration 2 times higher than that of use
	100g/L	Concentration 4 times higher than that of use
Tetraconazole	0.125mL/L	Concentration 4 times lower than the lowest concentration of use
	0.250mL/L	Concentration 2 times lower than the lowest concentration of use
	0.500mL/L	Concentration of use for grape cultivation
	0.750mL/L	Concentration of use for grape cultivation
	3,000 mL/L	Concentration 4 times higher than that of use

2.3 METHOD

This experiment was carried out in quadruplicate for each concentration of the actives, inside an exhaust hood and in the dark. Initially, the bulbs were placed in distilled water for 48 hours at room temperature to stimulate the development of the root meristem. After this period, the bulbs were placed in containers containing distilled water (negative control) and the test solutions. Each active ingredient was tested individually, according to the concentrations described in table 2.

The bulbs remained immersed in these solutions for a period of 72 hours, as described by Rank and Nielsen (11). After this period, a macroscopic analysis was performed, observing the morphological aspect of the roots, and then the length of the roots immersed in the test solutions was measured and compared with the negative control and the occurrence of cytotoxicity was considered when there was a statistically significant difference in the length of the roots submitted to growth in the test solutions in relation to the growth observed in the negative control. For this statistical evaluation, the Kruscal-Wallis test was used.

After measurement, the roots were collected and fixed in ethanol:acetic acid solution in the proportion of 3:1 for 6 hours and stored in 70% ethanol in a refrigerator (4ºC) for later cytogenetic analysis.

For the preparation of the slides, Feulgen staining was used, where the roots were subjected to acid hydrolysis with 1N HCl for 8 minutes at 60ºC, washed again in distilled water, stained with Shiff Reagent for 45 minutes in the dark, and then submitted to manual crushing on a glass slide for cytogenetic analysis. 2,000 cells of each replicate were analyzed

with the aid of a light microscope. The number of dividing cells observed in each reading was noted and later used to calculate the mitotic index (MI) by the following formula:

$$IM = \frac{number\ of\ cells\ in\ division}{number\ of\ cells\ counted}\ x\ 100 \qquad (1)$$

When any abnormality was observed in the cells during the reading, the abnormality was photographed using an optical microscope with a camera attached.

3 RESULTS AND DISCUSSION

Regarding the morphological aspect of the roots, the negative control presented the typical fasciculate root system, formed by adventitious roots with a healthy appearance and firm consistency. While the root system immersed in different concentrations of the test solutions also presented adventitious roots, but with a softer consistency. In general, as the concentration of the pesticide increased, the roots became more fragile, making it difficult to manipulate. Figure 1 shows the roots of *Alium strain* from the negative control and also from the different concentrations of test solutions of Provado 200 SC® (imidachlorid), but it should be noted that the other pesticides tested also showed similar macroscopic changes.

Figure 1

Evaluation of the development of the root meristem of A. cepa after immersion in containers containing distilled water as a negative control (a) and test solutions of Provado 200 SC® at different concentrations (b-f) for 72 hours at room temperature

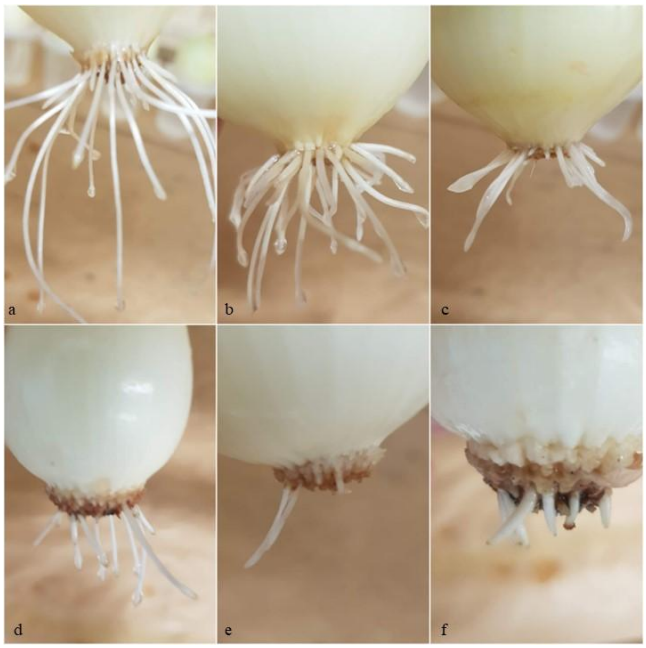


Figure 1 also shows the difference in the length of the roots of the negative control and the different concentrations tested, strongly suggesting that the pesticide has a cytotoxic effect. However, it is necessary to calculate the mitotic index, which is an indicator of the cytotoxic effect, as it is a marker of cell proliferation.

Table 3 presents the results of the mitotic index, as well as the number and length of the roots of all the active ingredients and their respective negative controls. All active ingredients, from the lowest concentrations, showed a reduction in root length compared to the negative control, presenting a statistically significant difference (p value less than 0.05).

Table 3

Mean size and quantity of roots and mitotic index for the different concentrations of the active ingredients compared to the respective negative controls (distilled water)

Active	Concentration	Average length (cm)	p-value *	Quantity	Mitotic index
Negative control	-	2,39	-	26,5	31,16%
They put in + pyraclostrobin	4g/L	1,1	< 0.05	30	31,61%
	8g/L	0,32	< 0.05	43	15,80%
	16g/L	0,92	< 0.05	43	1,32%
Active	**Concentration**	**Average length (cm)**	**p-value ***	**Quantity**	**Mitotic index**
Negative control	-	4,23	-	13,5	53,06%
Imidacloprid	0.2ml/L	1,33	< 0.05	13,5	50,46%
	0.25ml/L	1,86	< 0.05	7,75	45,02%
	0.40ml/L	1,3	< 0.05	6,25	39,61%
	0.50ml/L	1,07	< 0.05	5,25	34,85%
	8.75 ml/L	0,91	< 0.05	7	25,29%
Metalaxyl-M + Mancozeb	6.25g/L	1,85	< 0.05	6	14,39%
	12.5g/L	1	< 0.05	5,75	4,81%
	25g/L	1,09	< 0.05	6,5	3,50%
	50g/L	1,36	< 0.05	8,25	4,99%
	100g/L	1,44	< 0.05	6	0,00%
Active	**Concentration**	**Average length (cm)**	**p-value ***	**Quantity**	**Mitotic index**
Negative control	-	2,32	-	44	35,50%
Tetraconazole	0.125mL/L	0,95	< 0.05	14,5	33,21%
	0.250mL/L	0,9	< 0.05	19,25	31,35%
	0.500mL/L	0,82	< 0.05	26,5	30,46%
	0.750mL/L	0,9	< 0.05	19	21,26%
	3,000 mL/L	1,57	< 0.05	22,5	19,77%

Number of roots and size are presented as mean and the p-value, referring to the size of the roots, was calculated by the Kruskal-Wallis test.

Also in Table 3, most of the concentrations of the active ingredients tested showed a reduction in the mitotic index compared to the negative control and the higher the concentration of the active ingredient tested, the lower the mitotic index. The concentration

of metiram + pyraclostrobin use did not show a great difference in the percentage value of the mitotic index when compared to the negative control. However, when the concentration increased, the mitotic index reduced by 49.3% to 2 times the concentration of use and from 95.8% to 4 times the concentration of use.

For imidacloprid, the difference in the percentage value of the mitotic index when compared to the negative control was small (4.9%) for the first concentration of use, but increased to 15.2%, 25.3% and 34.3% in subsequent concentrations. This demonstrates the occurrence of cytotoxic effect at higher concentrations.

The farmer, when preparing the active ingredients for application in the crop, may come into contact with higher concentrations of the pesticide, and in this case, have a risk of poisoning if he is not properly using the Personal Protective Equipment (PPE).

For the active ingredients metalaxyl-m+mancozeb and tetraconazole, lower concentrations were also tested in relation to those used in viticulture. The lowest tested concentration of the active ingredient metalaxyl-m+mancozeb showed a mitotic index of 14.39%, a reduction of 72.9% in relation to the negative control, demonstrating to be a concentration with a high cytotoxic effect on *Allium cepa roots*. However, the lowest concentrations of tetraconazole presented percentage values of the mitotic index very close to those found in the negative control with a reduction of 6.5%. While for the highest concentrations of tetraconazole there was a decrease in the percentage values of the mitotic index in relation to the negative control, and for the highest concentration tested, there was a reduction of 44.3%. This decrease may probably be related to the action of this active ingredient in the suppression of DNA replication or to failure in the G2 phase of the cell cycle (12-14).

It is important to emphasize that the active ingredients selected for this study are all of a toxicological classification of low risk for acute poisoning, a fact that induces the farmer to handle these substances without due care, that is, without the proper use of the recommended PPE, it should be emphasized that the absence of risk of acute poisoning does not imply the absence of risk due to chronic exposure (15).

The fungicides Ridomil Gold-MZ® and Cabrio Top® are classified as unlikely to cause acute damage and little toxicity, respectively, but studies show that prolonged exposure to fungicides of the dithiocarbamates class, to which these fungicides belong, can lead to the appearance of respiratory allergies, dermatitis, or even more serious diseases such as Parkinson's disease and cancers (15).

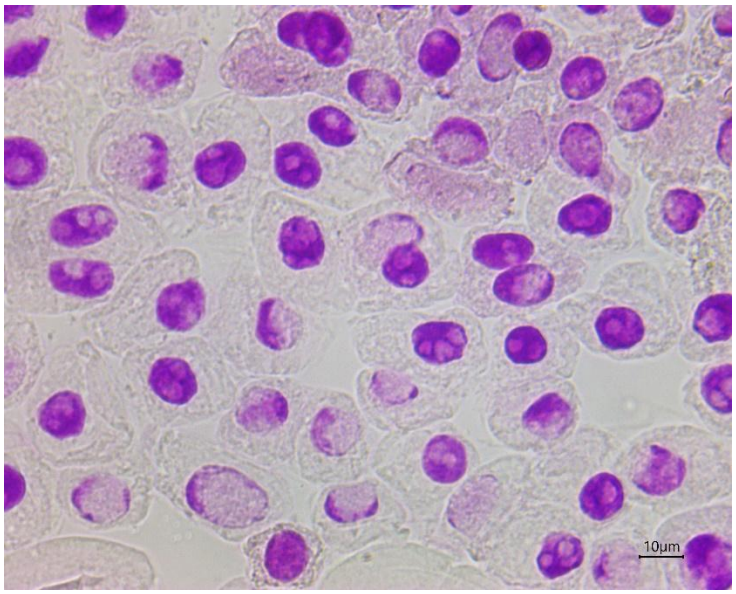
The package insert of Domark 100 EC® (tetraconazole) itself warns of liver damage due to chronic exposure. For Provado 200 SC® (imidacloprid) the effects of chronic exposure

are related to liver and thyroid decrease, as well as delayed bone calcification. This evidence was found in animal studies (16, 17).

Although there was no report of mutagenic or carcinogenic potential in the package insert of the substances tested(16-19). During the counting of cells that presented different phases of cell division, some abnormalities were observed in the genetic material such as binucleated cells, presence of nuclear vacuole and alterations related to the cell death process (pycnosis, karyorexis and karyolysis). Figure 2 shows the cells in the process of cell death and with nuclear vacuole.

Figure 2

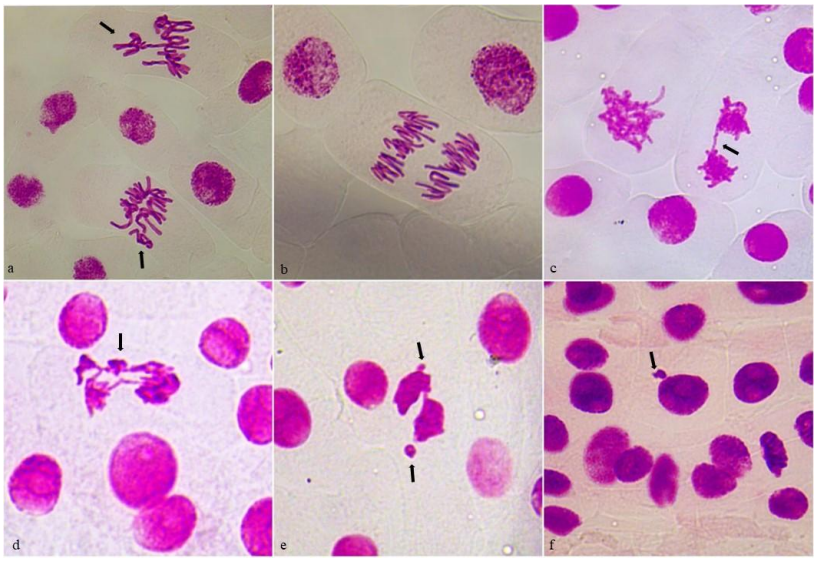
Allium cepa cells in the process of cell death after immersion in a Cabrio Top® test solution (Metiram + pyraclostrobin)



Also during the cell count, the occurrence of micronuclei, nuclear shoots, and chromosomal adhesion was observed. Metaphasic alterations such as c-metaphase, anaphasic alterations such as: anaphase bridge, multipolar anaphase, and telophasic alterations such as: telophase bridge; and other nuclear alterations such as pleomorphic nuclei. Figures 3 and 4 present images of some of the changes observed.

Figure 3

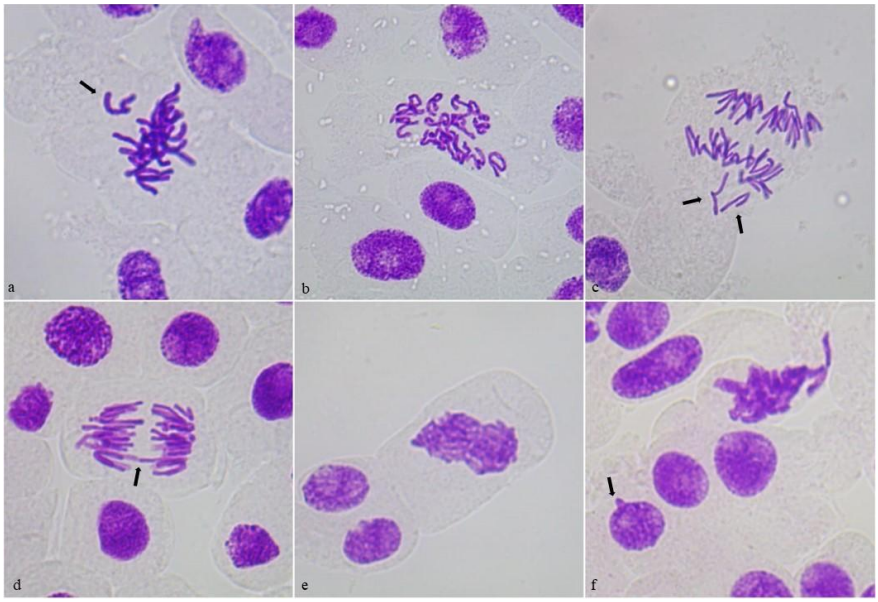
Abnormalities Observed in Allium Cepa Cells After Immersion in Test Solution of Provado 200 SC® (Imidacloprid)



a: upper arrow indicates cell with bridge in anaphase and with irregular division of DNA and lower arrow indicates genetic material outside the spindle in a cell in metaphase; b: cell in multipolar anaphase; c: arrow indicates telophase bridge and on the left side it is possible to observe a cell with chromosomal adhesion; d: arrow indicates two telophase bridges with pleomorphic nuclei; and: the arrows indicate micronuclei in a cell in telophase with pleomorphic nuclei; f: arrow indicates micronucleus in interphase cell.

Figure 4

Abnormalities observed in *Allium cepa cells* after immersion in Cabrio Top® (Metiram+pyraclostrobin) (a-d) and Domark 100 EC® (Tetraconazole) (e-f) test solution



a: arrow indicates genetic material outside the spindle in metaphase cell; b: c-metaphase; c: arrows indicate genetic material outside the spindle in a cell in multipolar anaphase; d: arrow indicates bridge in anaphase; e: telophase cell with adhesion; F: Arrow indicates nuclear bud in interphase cell and on the upper right side it is possible to observe a cell with adhesion, possibly in metaphase.

Specifically for the active ingredient imidacloprid, the occurrence of micronuclei, shoots, and chromosomal adhesion was mainly observed. But c-metaphase, bridge in anaphase, multipolar anaphase, and genetic material outside the spindle in metaphase and anaphase were also observed. Genotoxicity of imidacloprid has been reported in studies conducted with amphibians (20), human lymphocytes (21), HepG2 cells (22), fish (23), and plants (24). This genotoxic activity may be related to the fact that the active ingredient imidacloprid is a compound that has an electronegative pharmacophore, therefore, favoring the binding with the DNA molecule, and can generate genotoxic damage. This DNA damage can occur through oxidative stress that generates reactive oxygen species in large quantities, and is highly toxic to the body. Abnormal condensation of fibers leads to adhesion of chromosomal fibers, which can cause cell death or chromosomal abnormalities, such as micronuclei, for cases in which they persist until anaphase (25).

The active ingredient imidacloprid is used as an insecticide, showing efficacy in controlling pest insects. However, it is classified as harmful to the environment, and can be toxic to non-target organisms such as bees that are pollinators, and can also affect humans if used on a large scale (26-27).

The most frequently observed chromosomal abnormalities after the tetraconazole test were nuclear vacuole and chromosomal adhesion, which had a relationship between the increase in the occurrence of these abnormalities and the increase in the concentrations of the test solutions. The nuclear vacuole can lead to nuclear deformation, and its occurrence is indicative of suppression in DNA synthesis during the S phase of mitosis (28). Chromosomal adhesion is an irreversible abnormality caused by the degradation or depolymerization of chromosomal DNA, being associated with pesticides, and may even lead to cell death (29).

The occurrence of micronucleus was also observed with the increase in the concentrations of the test solutions, demonstrating that higher concentrations can present clastogenic or aneugenic effects, since micronuclei are described as resulting from these two basic phenomena in mitotic cells. Being formed by the acentric chromosomes or fragments of broken chromosomes or whole chromosomes or chromatids that delay in anaphase and are excluded from the daughter nucleus in telophase (30).

These chromosomal abnormalities may be related to the fact that tetraconazole is classified as a "potentially carcinogenic compound in humans", according to data from the Environmental Protection Agency (31). In addition to being a potentially carcinogenic compound, tetraconazole can trigger endocrine diseases in humans and animals by affecting the biosynthesis of steroid hormones (32).

Fungicides used to protect plants against fungal pathogens are widely used, but knowledge about the possible effects on organisms other than fungi is still insufficient (33). The bioassay using *A. cepa proved to* be a sensitive and reliable method, because by showing the occurrence of chromosomal abnormalities and micronuclei, it is a tool capable of indicating the clastogenicity of agrochemicals with a pesticidal effect (34). In addition, the determination of the mitotic index can be used as a bioindicator of cytotoxicity (35).

In this study, chromosomal abnormalities were observed. However, the genotoxic evaluation was not performed, through the calculation of the index of cellular abnormalities of the root meristem in contact with the test solutions and the negative control, in order to obtain more robust results in relation to the genotoxic effect.

Root growth may vary between different onion bulbs. In view of this situation, it is suggested that the contact of the bulb with the distilled water be maintained until the length of the root reaches 2 centimeters and not for a certain time (24 or 48 hours), so that a growth pattern can be maintained before the contact of the root with the active ingredient to be tested, allowing a better evaluation of cytotoxicity. The negative control should continue to be kept immersed in distilled water until the end of the test, as a guarantee that the conditions of the experiment did not influence the growth of the root.

The bioassay with *Allium cepa* showed cytotoxicity results for all the active ingredients tested, and the active ingredients imidacloprid and tetraconazole when used in concentrations higher than those indicated in grape cultivation promoted the formation of micronuclei and adherent chromosomes. This information is of great value, contributing to the development of additional research, using laboratory animals to relate cytogenotoxicity in humans. The results obtained also serve as a warning for rural workers to be made aware of the proper use of PPE during the handling and application of pesticides, especially those from family farming in the North Central Region of Paraná.

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