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In vitro toxicological prospection of fungicides containing Difenoconazole or Tebuconazole as active ingredients

Prospecção toxicológica in vitro de fungicidas contendo Difenoconazol ou Tebuconazol como ingredientes ativos

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Abstract

The active ingredients (AI) tebuconazole and difenoconazole (classified as toxic to living organisms and potentially mutagen, carcinogen, or teratogen) are part of the composition of widely used fungicides in crops. In 2016 they were found in irregular quantities by ANVISA in their latest report emitted by the Program of Analysis of Agrochemical Waste in Food. Two commercial fungicides containing the active ingredients tebuconazole and difenoconazole were evaluated in cytogenotoxic assays and on different biological molecules. Both active ingredients altered the clotting time of the plasma, and were procoagulants in the majority of the evaluated doses. The two AI acted on blood thrombi exerting thrombotic action and confirming the observed procoagulant potential. In the proteolysis assay, the AI did not alter the structure of fibrinogen under the conditions evaluated. Tebuconazole and Difenoconazole were also cytotoxic to human erythrocytes, as well as induced phospholipid breakdown, confirming their toxicity on membranes. However, under the conditions evaluated, the AI did not alter significantly

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the mitochondrial succinate dehydrogenase activity and did not induce DNA fragmentation. Assuming that changes in human cells and molecules have a cumulative effect, the toxic potential of fungicides might be greater when chronic exposure to their active principles occurs.

Keywords: Thrombolytic activity; Coagulant activity; Hemolytic activity; Proteolysis activity; Phospholipase activity.

Resumo

Os ingredientes ativos (IA) tebuconazol e difenoconazol (classificados como tóxicos aos organismos vivos e potencialmente mutagênicos, cancerígenos ou teratógenos) fazem parte da composição de fungicidas amplamente utilizados nas lavouras. Em 2016 foram encontrados em quantidades irregulares pela ANVISA em seu último relatório emitido pelo Programa de Análise de Resíduos Agroquímicos em Alimentos. Dois fungicidas comerciais contendo os princípios ativos tebuconazol e difenoconazol foram avaliados em ensaios citogenotóxicos e em diferentes moléculas biológicas. Ambos os princípios ativos alteraram o tempo de coagulação do plasma e foram pró-coagulantes na maioria das doses avaliadas. Os dois IA atuaram sobre os trombos sanguíneos exercendo ação trombótica e confirmando o potencial pró-coagulante observado. No ensaio de proteólise, o IA não alterou a estrutura do fibrinogênio nas condições avaliadas. Tebuconazol e Difenoconazol também foram citotóxicos para eritrócitos humanos, assim como induziram a quebra de fosfolipídios, confirmando sua toxicidade em membranas. No entanto, nas condições avaliadas, o IA não alterou significativamente a atividade da succinato desidrogenase mitocondrial e não induziu a fragmentação do DNA. Assumindo que as alterações nas células e moléculas humanas têm um efeito cumulativo, o potencial tóxico dos fungicidas pode ser maior quando ocorre exposição crônica aos seus princípios ativos.

Palavras-chave: Atividade trombolítica; Atividade coagulante; Atividade hemolítica; Atividade de proteólise; Atividade de fosfolipase.

Introduction

Brazil is the largest consumer of agrochemicals in the world (Frota & Siqueira, 2021). Only in 2019, more than 380 new agrochemicals were approved by the Ministry of Agriculture, Livestock, and Food Supply to be commercialized and used in crops. According to the Brazilian Health Regulatory Agency (ANVISA[a]), this is due to the widespread cultivation of monocultures, such as tomatoes, soybeans, wheat, barley, and corn. These compounds are efficiently used to control pests, pathogens, and invasive plants in crops, which ensures greater productivity and economic return (Handford *et al.* 2015). However, agrochemicals can cause impacts on the ecosystem once applied in agricultural areas because they can be leached, and, therefore, affect the groundwater and surface waters (Konwick *et al.* 2006; Souza *et al.* 2020). Consequently, they affect the surrounding biota, causing damage to aquatic and terrestrial organisms, including humans.

Some examples illustrate the damages to human health. In a study correlating exposure to pesticides and neurodegenerative diseases, an increase in the incidence of diseases, such as Alzheimer's, and even suicide attempts was observed to patients exposed to pesticides (Demur *et al.* 2013; Parrón *et al.* 2011). Another study, carried out *in vitro* with human cells, confirmed the endocrine-disruptive effect of agrochemicals mediated by estrogen receptors, androgen receptors, and aromatase activities (Kjeldsen *et al.* 2013; Pratush *et al.* 2020). Due

to misuse, it has been recognized in recent years the importance of elucidating the effects of these active ingredients on living organisms, and how they interact and contribute to the mechanisms of acute and chronic toxicity of fungicides.

Fungicides characterize one of the major classes of pesticides used in agriculture and can be classified as systemic or contact fungicides, or according to their chemical group: dithiocarbamates, benzimidazole, dicarboximides, and triazole (Benedetti *et al.* 2013). Triazoles are systemic fungicides formed by heterocyclic compounds with three nitrogen atoms in the same ring. Tebuconazole (TBZ) and difenoconazole (DFZ) are active ingredients that were reported by ANVISA in the last report of the Food Pesticide Residue Analysis Program (PARA) with values above the maximum residue limit for strawberry and grape, for example (ANVISA, 2016[a]).

Tebuconazole ((RS) - 1- p- chlorophenyl- 4,4- dimethyl-3- (1H-1,2,4-triazol-1-ylmethyl) pentan-3-ol) is a systemic active ingredient of the triazoles group whose function is to interfere in the metabolism of pathogenic fungi. As an example, the species *Fusarium culmorum* had its toxin production reduced when exposed to TBZ (Kang *et al.* 2001). The active ingredient act by decreasing the synthesis of ergosterol by the fungus when inhibiting enzymes of the cytochrome P450 (CYPS). The acceptable amount of ingestion of this fungicide is 0.02 mg kg^{-1} as determined by FAO (Food and Agriculture Organization of the United Nations) and WHO (World Health Organization) (FAO/WHO, 1994). Although it is considered safe for humans in low consumption (Muri et al. 2009), there is a concern about the use of this pesticide since there is accumulation of its effects and reports (both *in vivo* and *in vitro*) on their ability to cause malformations in animals (Menegola *et al.* 2013) and possible cancer in humans (Cui *et al.* 2018).

Difenoconazole (cis-trans-3-chloro-4-[4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl] phenyl 4- chlorophenyl ether) is also a fungicide of the triazoles family that has rapid action and prominent systemic activity (Dong et al. 2013). It is used in a variety of countries for its role in controlling a wide range of fungal diseases. DFZ mechanisms of action or toxicity are due to its ability to interfere with fungus growth and inhibit spore germination (activity very similar to tebuconazole). In addition, it can bind to proteins in various ways (e.g., hydrophobic interactions and hydrogen bonds), which leads to toxicity in humans (Li *et al.* 2013). According to ANVISA, its acceptable daily intake rate is 0.03mg kg^{-1} (ANVISA, 2016[b]).

Considering the exposed context, the aim of the present study was to evaluate two fungicides, with active ingredients belonging to the chemical group triazole (tebuconazole and difenoconazole), as to their genotoxic potential on human leukocytes, and their effects on cells and molecules mainly related to hemostasis. In order to do so, the comet assay and the coagulant / anticoagulant, proteolytic (substrates: fibrinogen and casein), thrombolytic, phospholipase, and hemolytic activities were used. In addition, their action on the mitochondrial activity was evaluated using the succinate dehydrogenase enzyme.

Material and Methods

Obtaining human biological material

The experimental protocols that required the use of human biological material were carried out in accordance with the recommendations of the Ethics Committee on Research on Human Beings (COEP) of the Federal University of Lavras (UFLA) (protocol number: 64335316.7.0000.5148). Blood samples were used in full or to obtain erythrocytes, leukocytes, or plasma.

Fungicides

Two fungicides were used in this study. Their active ingredients, chemical name, toxicity

class, and chemical formula are present in Table 1. The active ingredients (tebuconazole and difenoconazole) were selected because they are widely used in vegetable crops that are important to the state of Minas Gerais, especially in the tomato crop, since it stands out as the second largest producer in Brazil (IBGE/LSPA, 2015).

Solutions for the tests

Five concentrations of each active ingredients were used to perform the tests. The solutions were prepared by diluting the active ingredients in water according to instructions found on the commercial product leaflet. The concentrations tested are identified in Table 2 and were based on the ones recommended for the tomato crop.

Table 1. Characterization of tebuconazole and difenoconazole according to ANVISA.

Active Ingredient	Chemical name	Toxicity Class	Chemical Formula
Tebuconazole[a]	(RS)-1-p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl) pentan-3-ol	IV - Slightly Toxic	$C_{16}H_{22}ClN_3O$
Difenoconazole[b]	cis-trans-3-chloro-4-[4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether	I - Extremely toxic	$C_{19}H_{17}C_{l2}N_3O_3$

References according to ANVISA's data (Anvisa 2016[b]; Anvisa 2016[c])

Table 2. Concentrations of the active ingredients tebuconazole and difenoconazole (µg mL^{-1}), according to the tolerated limit by ANVISA and the concentrations recommended by the manufacturers for the tomato crop.

Concentrations	Ratio to the recommended	Active Ingredients [µg mL^{-1}]	
		Difenoconazole	Tebuconazole
1/4x	¼	31.25	25
1/2x	½	62.5	50
R	recommended	125	100
2x	2 times higher	250	200
4x	4 times higher	500	400

Genotoxic activity on human leukocytes - Comet Assay

The methodology of Singh et al. (1988), adapted following the protocol described by Olive and Banáth (2006), was used for the comet assay in alkaline conditions. The tests were performed using whole blood, with subsequent analysis of only the leukocyte nucleoids. The cells (500 µL of blood plus 500 µL of PBS) remained in the presence of the treatments (difenoconazole and tebuconazole, in different concentrations) for four hours in a cell culture chamber at 37 °C, in order to evaluate the immediate effects of an exposure to the fungicides. 15 µL of each incubation solution were mixed with 100 µL of LMP (low melting point) agarose, then pipetted onto the

slide (previously covered with 1% NMP -normal melting point- agarose) and covered with a cover slip. Then, the coverslips were removed, and the slides were dipped in lysis solution (0.25 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 adjusted with 10 M NaOH, 1% Triton X-100, 10% DMSO) where they remained for 12 hours. The positive (C+) control was performed using doxorubicin - genotoxic antitumor drug (50 μg mL^{-1}) and the negative control (C-) was PBS. Before undergoing electrophoresis (30 min at 25 V), the slides were transferred to an electrophoresis solution (1 mM EDTA, 300 mM NaOH, pH 13) where they remained for 25 min at 4 °C. After the electrophoresis run, the slides were placed in a neutralization solution (0.4 M Tris-HCl, pH 7.4) for 30 min. The DNA contained in the slides was precipitated with absolute ethanol and then dried at room temperature.

The staining was performed with propidium iodide at 2 μg mL^{-1}. All steps of this assay were performed in the dark. Comet patterns were analyzed by visual scores on an epifluorescence microscope (Olympus BX51), according to Collins et al. (1997), in which the analyzed nucleoids were classified as to the extent of the lesion in DNA.

Activity on human plasma coagulation

The effects on coagulation were evaluated according to the methodology described by Mourão et al. (1996). The treatments were added to citrated human plasma (200 μL), stabilized at 37 °C, with gentle stirring and constant observation until clot formation. The compounds potential to inhibit coagulation was evaluated by adding the treatments to citrated plasma (incubation of 10 min) and subsequent addition of 50 μL of a 100 mM calcium chloride solution. The time required for the formation of the clot was measured in seconds. When compared to the control containing only $CaCl_2$, reducing the clotting time was considered as pro-coagulant activity and the increase of time as anticoagulant activity.

Activity on fibrinogenolysis

The methodology of polyacrylamide gel electrophoresis (PAGE) under reducing conditions, described by Edgar and Prentice (1973), was used to evaluate possible interactions between the active ingredients and fibrinogen molecules. The compounds were incubated with the protein molecules in different proportions for 2 hours at 37 °C. Afterwards, the samples were denatured with the addition of β-mercaptoethanol and placed in a boiling bath for 5 min. The samples were analyzed in a 12% polyacrylamide gel (m / v), that allows the observation of the α, β, and γ chains of the fibrinogen (control), as well as the presence of fibrinopeptides in the samples in which proteolysis occurs. The venom of *Bothrops atrox* (60 μg), which presents fibrinogenolytic enzymes in its composition, was used as positive control.

Activity on human blood thrombi

The thrombolytic activity was evaluated on blood clots formed *in vitro* according to the methodology described by Cintra et al. (2012). Each thrombus was obtained by dispensing 100 μL fresh blood into each well of microtiter plates and allowing it to coagulate for 15 min at room temperature. After this period, the clots were incubated for 24 hours at 37 °C with different concentrations of the compounds, using a thrombolytic agent (*Bothrops atrox* venom, 30 μg) and PBS as positive and negative controls, respectively. After incubation, the thrombolytic activity was assessed by measuring the volumes of fluid released from the thrombi.

Effects on the phospholipase A_2 activity

The phospholipase activity was performed according to the methodology described by Gutiérrez et al. (1988), with a few adaptations. A medium was made

with 0.01 mol L^{-1} $CaCl_2$, egg yolk lecithins (phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine) 0.005% sodium azide (inhibits the growth of microorganisms), and 1% bacteriological agar dissolved in PBS (pH 7.2). The medium was poured into petri dishes and, after its solidification, holes of uniform size (~ 0.5 cm in diameter) were made and the treatments applied into them. The translucent halos, resulting from phospholipase activity (expressed in millimeters), were evaluated after 18 hours of incubation of the plates in a cell culture chamber at 37 °C. *B. atrox* (10 µg) venom was used as a positive control.

Cytotoxicity evaluation on human erythrocytes (solid medium)

This activity was evaluated using a solid media as described by Gutiérrez et al. (1988), but replacing the egg yolk phospholipids with a concentrate of human erythrocytes that gives a hematocrit of approximately 1%. After blood collection in tubes containing heparin, it was immediately centrifuged and the supernatant discarded. The cells were suspended in PBS buffer and then washed twice in 1200 *g* centrifugations for 10 minutes. After centrifugation, the obtained erythrocyte concentrate was used in the gel composition.

The gel was made (0.01 mol L^{-1} $CaCl_2$, erythrocytes washed in PBS 1:3 v v^{-1}, and 0,005% sodium azide) with 1% bacteriological agar dissolved in PBS, pH 7.2. The medium was poured into petri dishes and, after their solidification, holes of uniform size were made (~ 0.5 cm in diameter) and the treatments applied. The effects of the treatments and the PBS (negative control) were evaluated after the plates stayed in a cell culture chamber for 18 hours at 37 °C. The formation of a translucent halo around the hole in the gel characterizes the hemolytic activity, which is represented by the measurement (in millimeters) of the halo diameter.

Cytotoxicity evaluation on human erythrocytes (liquid medium)

To obtain the erythrocytes used in this activity, 6 mL of blood was collected in heparin-containing vacutainers and the volume filled with PBS (2 mM NaH_2PO_4, 3 mM Na_2HPO_4, and 154 mM NaCl - pH 7.4) to obtain 12 mL. Then, the blood was centrifuged 3 times for 5 minutes at 700 g (Fanem Baby® Model 206 BL), and the supernatant replaced with PBS at each replicate. The erythrocytes were diluted to obtain hematocrits of 0.5% and 1%.

The erythrocytes were added to microtubes along with the samples (tebuconazole, difenoconazole, PBS - mechanical hemolysis control - and distilled water - total hemolysis control) and incubated at 37 °C for 1h (homogenized every 10 min). After the incubation period, centrifugation was carried out for 5 min at 1500 *g* and spectrophotometry were performed at 412 nm according to the methodology described by Rangel et al. (1997), with few modifications (Preté et al. 2011).

The percentage of hemolysis was calculated based on the concentration of hemoglobin present in the supernatant of the incubates, according to the following equation: % hemolysis = $A_{am} - A_{PBS} / A_{H2O} - A_{PBS} \times 100$, in which A_{am}, A_{PBS}, and A_{H2O} are the absorbance of the test samples, the PBS as control, and the water as control, respectively.

Activity of mitochondrial succinate dehydrogenase

In this experiment the objective was to evaluate the possible inhibitory action of the active ingredients on the activity of the enzyme succinate dehydrogenase (acts on the Krebs cycle and the electron transport chain).

Obtaining the mitochondrial extract

Fresh chicken liver was homogenized (30 seconds at 4 °C) in a blender using 100 mL of 0.25 M sucrose in 0.05 M phosphate buffer, pH 7.4. Thereafter, the material was filtered in gauze and centrifuged at 3300 *g* for 10 minutes at 4 °C. The supernatant was discarded and the sediment enriched with mitochondria was used as the source of the enzyme succinate dehydrogenase.

Evaluation of the effect of the active ingredients on the activity of the enzyme succinate dehydrogenase

The mitochondria were incubated (30 minutes at 37 °C) with Difenoconazole or Tebuconazole at different concentrations, according to Table 2. After this incubation, a solution of sodium succinate (enzyme substrate) and a solution of 0.5 mL of 0.05M KCN prepared in 0.1 M sodium phosphate buffer (pH 7.4) and 2.0 mL of 0.0004 M dichlorophenol (DCP) were added to the tubes, followed by further incubations of 15 and 30 minutes. Afterward, inside an ice bath, 0.2 mL of 0.3 M malonic acid (enzyme inhibitor), pH 7.4, was added to all tubes to interrupt the activity of succinate dehydrogenase. The analyses were taken at 630 nm at the spectrophotometer.

The oxidation of succinate to fumarate and reduction of FAD^+ to $FADH_2$ occurs in the presence of the active enzyme. The $FADH_2$ donates an electron pair to DCPI forming FAD^+ and DCPI-H_2. The graph profile of the absorbance reading of the formed product follows the Lambert-beer equation (**A = E. b. c**), in which **E** (molar absorption coefficient) corresponds to the absorptivity of DCPI at the wavelength of 600 nm – equivalent to 21.500 $\mu mol.L^{-1}.cm^{-1}$ (Bardaweel et al. 2011), **b** is the path length, and **c** is the concentration - the enzyme activity itself in U. mL^{-1}.

The percentage of enzyme activity was calculated by comparing with the activity of the enzyme without inhibitor and its was considered as 100% activity.

Statistical analysis

The obtained data were submitted to the Shapiro-Wilk test ($p<0.05$) to verify the normality of the data. The Dunnet test was performed to the data that had normal distribution. The data that did not present normal distribution, an attempt to normalize by the PROC RANK procedure was performed. The non-normalized data were compared by the KrusKal-Wallis test ($p<0.05$). The statistical program used was SAS (9.0).

Results

In this work, we evaluated the performance of the two triazoles (tebuconazole and difenoconazole) using the comet assay. There were no significant levels of fragmentation observed at the concentrations used in the nucleotides obtained from human blood leukocytes (data not shown), probably due to the genotoxic/ mutagenic effects of these compounds being conditioned to cumulative and chronic effects.

The concentrations used here were based on the dilution recommended by the manufacturers for the use and application of the studied fungicides (Table 2), and, when compared with the data in similar studies they do not differ.

In this experiment no breaks or changes in the protein migration profile were observed in comparison to the control. Although not observed by electrophoresis, the possible mild bind to fibrinogen may be enough to induce undesired immune responses.

The concentrations determined for our work did not induce hemolysis in solid medium and low hemolysis in liquid medium. The formation of halos were observed in the concentrations of 12.5 mg mL^{-1} (100x higher than the recommended) and 125 mg mL^{-1} (1000x higher than the recommended) in the hemolytic activity in solid medium, with diameters of 7 mm and 10 mm, respectively. On the other hand, difenoconazole caused hemolysis halos only at the concentration of 1000x (100 mg mL^{-1}) higher than the

recommended, with a diameter of 9.3 mm.

In the evaluation of hemolysis in liquid medium (Table 3), it was observed statistically significant percentages of lysis of erythrocytes, in both hematocrits evaluated, when they were treated with tebuconazole at 100.000 μg mL^{-1}. In addition, hemolysis was observed at concentrations from 250 μg mL^{-1} to 125.000 μg mL^{-1} when treated with difenoconazole.

In the coagulant activity (Table 4), there was a decrease in the clotting time in the plasma samples treated with both active ingredients (compared to the positive control). However, this occurs in different proportions for the AI evaluated, since difenoconazole reduced clotting time to a greater extent at the concentration of 500 μg mL^{-1}, whereas tebuconazole induced a greater procoagulant effect at the concentrations of 25 and 50 μg mL^{-1}. Although the results found in the statistical analysis confirm only the activities of these three concentrations, it is not wise to disregard the others that have procoagulant potential.

The results found in the thrombolytic activity

Table 3. Hemolytic activity evaluated in liquid medium, in 0.5% and 1% hematocrits, after treatment with the active ingredients difenoconazole and tebuconazole.

HEMOLYSIS PERCENTAGE					
Difenoconazole	**Hematocrit**		**Tebuconazole**	**Hematocrit**	
[μg mL^{-1}]	**0.5%**	**1%**	[μg mL^{-1}]	**0.5%**	**1%**
31.25	n.a	n.a	25	n.a	n.a
62.5	n.a	n.a	50	n.a	n.a
125	n.a	n.a	100	n.a	n.a
250	5.22%	0.83%	200	n.a	n.a
500	2.33%	2.35%	400	n.a	n.a
12.500	100%	60.86%	10.000	n.a	n.a
125.000	100%	100%	100.000	77%	38%

The positive control (H_2O-induced osmotic lysis) was considered as 100% hemolysis. n.a (no activity). The data represent the averages of triplicates obtained in 3 independent experiments.

Table 4. Coagulant activity evaluated in human citrated plasma in the presence of the active principles tebuconazole and difenoconazole.

PLASMA CLOTTING TIME (seconds)					
	Difenoconazole		**Tebuconazole**		
	[μg mL^{-1}]	**Time**	**[μg mL^{-1}]**	**Time**	
C + 126	31.25	98.33 b	25	88.67 b	**C + 120**
	62.5	151.33 a	50	76.33 b	
	125	100.33 b	100	138.33 a	
	250	99.67 b	200	108 b	
	500	86* b	400	129.3 a	

C+ (positive control): $CaCl_2$ 0.1M. The data represent the averages of the clotting time (seconds) in triplicates, obtained in 3 independent experiments. **a:** differs from the respective positive control in Anti-coagulant potential. **b:** differs from the respective positive control in Procoagulant potential.

Table 5. Thrombolytic activity on human blood thrombi assessed after the treatment with the active ingredients difenoconazole and tebuconazole.

QUANTIFICATION OF LYSIS BY THE VOLUME OF LIQUID RELEASED FROM THE THROMBI (µL)				
Difenoconazole		**Tebuconazole**		
[µg mL^{-1}]	**Volume**	**[µg mL^{-1}]**	**Volume**	
31.25	35.83 a	25	38.16 a	**C +**
62.5	44 a	50	37.33 a	**55**
125	40.33 a	100	39 a	
250	40.83 a	200	31.33 a	**C -**
500	45.83 a	400	40.5 a	**39.3**

C+: *B. atrox* venom (30 µg). C-: PBS. The data represent the averages of the volumes released by the thrombi, in triplicates - obtained in 3 independent experiments. **a:** differs from the positive control in prothrombotic potential.

assay (Table 5) demonstrate a lower dissolution of the thrombi by the active ingredients than the one observed for the positive control (*Bothrops atrox* venom, 30 µg), whose composition presents hemorrhagic proteases and PLA_2s with anti-platelet and hemolytic action. Both tebuconazole and difenoconazole presented results that are statistically different from the positive control ($p<0,05$). Some concentrations presented a prothrombotic potential (Table 5), which means they make the thrombi more rigid and reduce the release of liquid through the thrombus. This information corroborates with the procoagulant effect observed in this study (Table 4).

The results obtained for the phospholipase activity (Figure 1) show that all concentrations tested for both active ingredients presented statistical similarity ($p < 0.05$) to the positive control (*B. atrox* venom, 10 µg).

Discussion

In 2001, the Brazilian government created the Food Pesticide Residue Analysis Program, PARA. This program aims to continuously assess the levels of pesticide residues in food of plant origin that are intended for the consumer. ANVISA (the agency that regulates the program) analyzes more than 25 types of food, and so far more than 30 thousand analyses have been carried out. The latest report is from the triennium 2013-2015 and provides data on the risk related to the consumption of these foods (ANVISA, 2016[c]). The main problem found in the analyzed samples is the indiscriminate use of unauthorized pesticides in crops, followed by the high residue levels in the samples.

However, the project PL 6922/2002, which is related to a set of measures that aims to make the approval and implementation of agricultural pesticides more flexible, has been advancing in the Legislative Assembly for sanction. Given the imminence of the risks associated with the approval of this bill, scientific research is necessary to understand the effects of pesticides that are currently in use.

Both active ingredients (tebuconazol and difenoconazol) have already been evaluated by several authors regarding their toxicity, but few are the works in which the objective of the study are the damages caused in humans. Most organisms in which TBZ and DFZ have been tested are aquatic (ANVISA, 2016[a, b]).

Since the 1990s, Brazil has been closing partnerships with the Organization for Economic

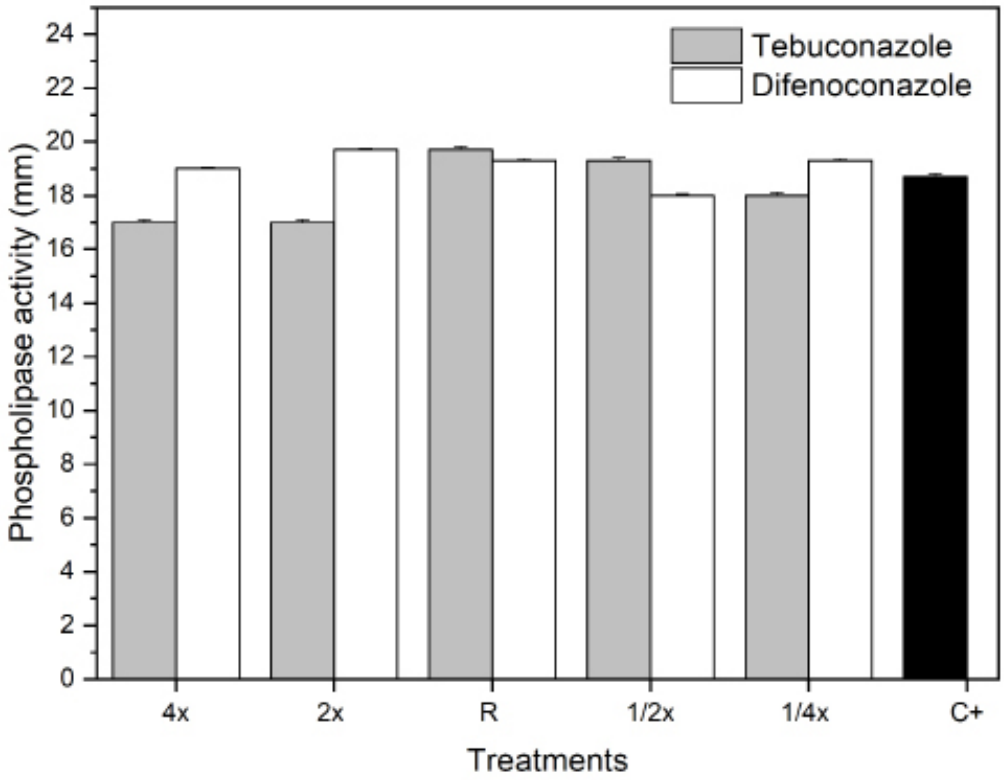

Figure 1. Phospholipase activity assessed after the treatment of phospholipids with difenoconazole and tebuconazole. C+: *B. atrox* venom at 10µg. C-: PBS. The data represent the averages of the diameters of the halos (mm) obtained in 3 independent experiments.

Co-Operation and Development (Buschmann & Totowa, 2019) in order to pair its regulatory measures based in the ones implemented in developed countries. The comet assay is among the main regulatory tests required by the organization for the evaluation of the genotoxic potential of substances (Buschmann & Totowa, 2019).

A study conducted in 1998 by Lebailly and coworkers in a farming region of France measured the effects of a single exposure to various classes of agrochemicals (Lebailly et al. 1998). The triazoles group, represented by epoxiconazole and cyproconazole, did not present statistically significant effects. A second study, conducted by Schwarzbacherová and Šiviková (2015), evaluated the effect of a 24 and 48 exposure on bovine lymphocytes. The fungicide Prosaro® (tebuconazole / prothioconazole) was not considered toxic, except for the concentration of 30 µg mL^{-1}. The concentrations used in this study did not have similar effects due to the time of exposure (four hours).

In contrast to the above studies, the work conducted by Baurand et al. (2015), in which the authors evaluated the genotoxicity of certain pesticides, including tebuconazole, significant changes were noted only at concentrations lower than those recommended by the manufacturer.

The comet assay conducted with Zebrafish (*Danio rerio*) and tebuconazole at concentrations of 100, 200, and 300 µg L^{-1} presented genotoxic action (Castro et al. 2018). The similarity between the human and fish cells allows the comparison of the studies. Considering the longer exposure time (24, 48, and 72 hours) for fish cells, it is suggested a re-evaluation of the fungicides activity increasing their exposure time on human cells. To support this suggestion, Martinez et al. (2014) used the comet assay to evaluate the action of tebuconazole on cells of two species of green algae (*Pseudokirchneriella subcapitata* and *Nannocloris oculata*), exposed for 24 hours. The

active ingredient was genotoxic when tested at the concentrations of 3 and 6 mg L^{-1}. Confirming the genotoxicity observed for tebuconazole, Siviková et al. (2013) evaluated the fungicide at the concentrations of 3, 6, 15, 30, and 60 μg. mL^{-1} using the chromosome aberration test, the sister chromatid exchange assay, the micronucleus assay, and the mitotic index. Given the contrasting results obtained in the literature, it is important to conduct new experiments using other models to better evaluate the mutagenic effects of the triazoles used herein. We suggest, therefore, the use of chronic toxicity models and in different cell types, such as nerve or sexual cells.

One of the concerns while using agrochemicals is their action when in contact with plasma proteins. Recent studies have demonstrated the ability of tebuconazole to bind to albumin (Staničovájana et al. 2018). It is known that interactions of this nature can affect the metabolism, excretion, and permanence of such agents in the body. In addition, plasma proteins with altered behavior may trigger undesired immune responses and pose a risk to the health of the affected individuals.

As the ability to moderately bind to albumin is known, our research group has evaluated the possibility of tebuconazole and difenoconazole on binding to fibrinogen, and to verify the induction of breaks or modifications in the migration profile of this protein. In the search for better results in the crop many farmers choose to use the fungicides in doses above the recommended, or apply them continuously for long periods, and this can be a high risk to people who are in contact with these agents. In this way, larger doses were tested in order to observe the effect of the active principles on the hemolytic activity.

According to the parameters proposed by ASTM International, substances with values greater than 5% of hemoglobin release are considered as hemolytic (ASTM, 2017). Based on the results obtained in this present study, only excessively high doses (above 2x the recommended value) were able to induce a significantly high hemolysis.

Due to the experimental conditions and the relevance of a few seconds in the development of the coagulation cascade, the concentrations of 31.25, 125, and 250 μg mL^{-1} for difenoconazole and 200 and 400 μg mL^{-1} for tebuconazole were also considered as procoagulant (Table 4). This evaluation was performed by giving 25 seconds of difference between the control and the treatments, since this time is enough to trigger some processes of the coagulation cascade (e.g., an activation of prothrombin - usually between 10 and 14 seconds- and partial activation of thromboplastin - usually between 24 and 40 seconds) according to a coagulogram data.

By reducing the clotting time, both difenoconazole and tebuconazole can be considered as procoagulants. Considering the presence of these compounds within the human body it is possible to suggest their action in the formation of clots/ thrombi within the bloodstream, generating pathological conditions such as thrombosis and ischemia.

The evaluated compounds are toxic since they induce the breakdown of phospholipids (Figure 1). Thus, they may have action on cell membranes (Koeppen & Stanton, 2009) by causing changes in the transport of molecules and ions. They may also affect processes such as blood coagulation (Tortora & Derrickson, 2017) and inflammatory response closely related to degradation of phospholipids (e.g., degradation of arachidonic acid that results in the synthesis of prostaglandins, thromboxanes, and leukotrienes via cyclooxygenases and lipoxygenases). The predominant phospholipids in the outer monolayer of human erythrocyte membranes are phosphatidylcholine and sphingomyelin, whereas in the inner monolayer phosphatidylethanolamine and phosphatidylserine predominate (Yawata, 2003).

Under the evaluated *in vitro* conditions, the activity of the succinate dehydrogenase enzyme added to the active ingredients (TBZ and DFZ) was not statistically different from the negative

control (enzyme + substrate) (data not shown). However, it is necessary to consider the re-evaluation of the test under different conditions to better observe the possible effects of these compounds, since the behavior of the enzyme, the molecular interactions, and the time required to observe the effects may differ *in vivo*. In addition, it is suggested to study other enzymes related to the Krebs cycle or electron transport chain (complex II), in order to define the possible toxic effects of these active ingredients.

The effect of some fungicides (one of them being difenoconazole) on the mitochondria of the flight muscle of bees (*Bombus terrestris*) was evaluated in a study by Syromyatnikov et al. (2017). The authors reported that difenoconazole inhibited the mitochondrial respiration at the level of complex I and glycerol-3-phosphate dehydrogenase in the electron transport chain.

Although fungicides have proven to be effective against phytopathogenic fungi (increasing productivity and economic returns to farmers), they can have adverse effects on many organisms when widely used in uncontrolled and unregulated amounts. As evidenced by Mercadante et al. (2014) in a study evaluating and quantifying tebuconazole residues in human urine of rural workers, this compound was found in amounts of 10 to 473 μg L^{-1} (TEB-OH) and 3 to 159 μg L^{-1} (TEB-COOH). Other experiments in this area have demonstrated that the metabolites TEB-OH and TEB-COOH, because they are excreted in the urine, are good biomonitors of the presence of tebuconazole in the human organism (Fustinoni et al. 2014). Therefore, with the accumulation within the human body demonstrated by previous studies and the results obtained in the present work, the human health risks related to the use of the fungicides containing the active ingredients difenoconazole and tebuconazole are proven.

Conclusion

Both fungicides containing the active ingredients tebuconazole and difenoconazole induced hemostasis disorders, presented cytotoxicity on human erythrocytes, and were able to degrade phospholipids that are widely distributed in cell membranes. For a better evaluation of its genotoxic and mutagenic potential the comet assay should be evaluated at different times of exposure to the active ingredients. It is worth noting that the lower concentrations of tebuconazole were more effective in causing changes during the tests performed, whereas for difenoconazole the changes were in the highest concentrations. Complementary studies should be carried out to determine new concentrations for the use of the fungicides, since the induction of changes in human cells and molecules has been proven to occur even in concentrations lower than those recommended by the manufacturers. In addition, it is suggested to study the mutagenic and teratogenic action of these active ingredients in humans, since the farmers work with multiple exposures to pesticides in their plantations, and do not always make use of PPE (Personal Protective Equipment). Thus, the accumulation of the effects of difenoconazole and tebuconazole on the organism can result in even more relevant changes than those found in this work.

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Conflict of interest

The authors have declared that there is no conflict of interest.

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