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Exposure of *Vicia faba* to sulcotrione pesticide induced genotoxicity

Chaima Sta^{a,b}, Gérard Ledoigt^{a,*}, Ezzeddine Ferjani^b, Pascale Goupil^a

^a Clermont Université, Université Blaise Pascal, UMR 547 PIAF, BP 10448, F-63000 CLERMONT-FERRAND, Campus Universitaire des Cézéaux, 24 Avenue des Landais, 63177 Aubière cedex, France

^b Laboratoire de Physiologie et Génétique des Plantes à Intérêt Agronomique, Faculté des Sciences de Bizerte, Université de Carthage, 7021 Jarzouna, Tunisia

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ABSTRACT

Potential genotoxicity of sulcotrione 2-(2-chloro-4-(methylsulfonyl)benzoyl)-1,3-cyclohexanedione, a selective triketone herbicide was evaluated on *Vicia faba* seedlings in hydroponic culture conditions. Sulcotrione (10^{-5} , 10^{-4} and 2×10^{-4} M) treatments for 45 h, caused a dose dependent increase in micronuclei frequencies in root meristematic cells. Cytological analysis of root tips cells showed aneugenic effects of the sulcotrione on the plant root meristems. Sulcotrione induced chromosomal alterations at the lowest concentration used (10^{-5} M) when incubated for 42 h, indicating the potent mutagenic effect of this element. This is the first report for the genotoxicity of such a sulcotrione herbicide.

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1. Introduction

Sulcotrione, chemically defined as 2-(2-chloro-(4-methylsulfonyl)benzoyl)-1,3-cyclohexanedione (Fig. 1) is a recent triketone herbicide used to control a wide range of grasses and broad leaf weeds in corn crop yields and is proposed as an atrazine substitute. In target plant species, the action of this herbicide (Fig. 1) consists in the inhibition of the enzyme 4-hydroxyphenyl pyruvate dioxygenase (p-HPPD) [1,2] leading to strong bleaching effects accompanied by a decrease in chlorophyll and carotenoid levels as well as by a massive accumulation of phytoene [3,4], necrosis and death of sensitive plants [5]. Sulcotrione is an herbicide applied at a rate of 450 g ha^{-1} at maize post-emergence. It is absorbed by the leaves and also by the roots [6,7]. Triketones herbicides inhibit the chain of photosynthetic electron transfer that is blocking a vital mechanism of energy production within the plant [8]. The application of these active ingredients should be at an early stage of development of dicotyledonous targeted to be fully effective and lead to their removal. The triketones act by foliar and root channels and have an action antigerminative to control weeds after treatment.

Cellular metabolism of sulcotrione produces CMBA (2-chloro-4-methylsulfonylbenzoic acid) in plants and hydroxysulcotrione in mammals. Sulcotrione is persistent for a short time but mobile [6,7,9]. The photodegradation of sulcotrione leads to a stable toxic

intermediate product that is prevented by using grape natural products [10–14].

Extensive use of herbicides in agriculture and potential carcinogenicity strongly emphasize the need to extend the genotoxic evaluation of these compounds by using different assays. Mitotic root meristems of *Vicia faba* have been pioneer cytogenetic materials for genotoxicity studies of physical and chemical agents since the early 1930s. It has a low chromosome number ($2n = 12$), making it suitable for cytogenetic studies. The formation of micronuclei (MCN) in root tips has been widely described and used as a bioassay for the evaluation of *in vivo* mutagenic effects of environmental pollutants on plants and animals [15–17]. The increased frequency of micronuclei was observed in many aquatic organisms exposed to various pollutants and indifferent cell types. Among biomarkers of exposure measured in animals, frequency of micronuclei was the one best correlated with the pollutant load in tissue. Several authors [18] concluded on the ability of this tool to reveal chronic contamination by persistent genotoxic agents in water and/or sediment at low concentrations.

Micronuclei can be composed of small chromosome fragments resulting from chromosome breaks caused by clastogenic activity. The failure of entire chromosomes to migrate during anaphase as a result of the aneugenic effects of genotoxic agents also can lead to micronucleus formation [16,18–21].

In this study, we first reported the potentially genotoxic effects of sulcotrione for meristematic root cells of a Fabaceae species, *V. faba*. Cytological analysis of *V. faba* meristematic root cells showed the potential aneugenic effects of the pesticide on plant chromosomes.

Abbreviations: MCN, micronucleus; MH, maleic hydrazide; MI, mitotic index.

* Corresponding author. Fax: +33 473407942.

E-mail address: gerard.ledoigt@univ-bpclermont.fr (G. Ledoigt).

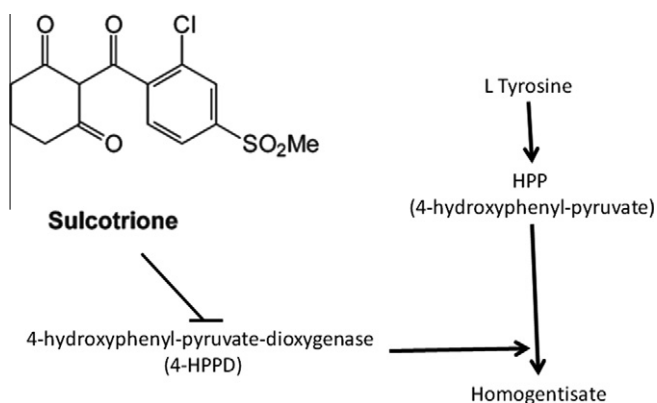


Fig. 1. Molecular structure of sulcotrione and its physiological herbicide effect on HPPD.

2. Materials and methods

2.1. Plant material and chemicals

Sulcotrione [2-(2-Chloro-4-(methylsulfonyl) benzoyl)-1, 3-cyclohexanedione], 98.7%, Mm 328.77] was purchased from Riedel de Haën (Pestanal[®], Saint-Quentin Fallavier, France).

Seeds of *V. faba* var. Aguadulce were surface sterilized with 10% sodium hypochloride, rinsed several times with distilled water and placed on moistened paper at 25 °C for 4–5 days, allowing them to germinate and transferred to a hydroponic support in the following nutrient solution (pH 7, 3.9 mM Ca(NO₃)₂, 6.5 mM KNO₃, 2 mM MgSO₄, 0.9 mM KH₂PO₄ plus micro-nutrients: 90 μM Fe-ethylene diamine tetra-acetic acid, 2.7 μM MnSO₄, 0.8 μM ZnSO₄, 4.5 μM H₃BO₃, 4 μM CuSO₄ and 2.0 μM Mo₇O₂₄(NH₄)₆) as described by Souguir et al. [18]. Culture takes place in air-conditioned room under controlled conditions: 16 h light/8 h darkness (Mazdafluor Prestiflux) at a temperature 25/20 °C and a relative humidity of 65% (±5%).

2.2. Plant treatments

Once roots had reached a length of 2–3 cm, additional concentrations of sulcotrione (10^{-5} – 2×10^{-4} M) were added to the hydroponic solution for 45 h at 25 °C with a light/dark photoperiod of 16:8 h. All tests were repeated six times, using a negative control (SNS, containing no additional sulcotrione), and nutrient solution containing 4×10^{-3} M maleic hydrazide (MH) as a positive control. MH is a herbicide known to be a mutagenic and clastogenic agent [22]. Maleic hydrazide is chemically defined as 1,2-dihydro-3,6-pyridazinedione, an uracile isomer, and a herbicide used in agriculture. It is known for its ability to induce micronuclei and chromosomal aberrations in *V. faba* [22].

V. faba root tip micronucleus test is one of the most employed plant genotoxicity assays, and has been used on various types of contaminated materials. A study of the effect of inorganic pollutants, such as cadmium, on bean roots in hydroponic culture, showed a highly significant difference in the mitotic index ($P < 0.001$) at 48 h of treatment [23], therefore we have chosen a similar period of treatment with pesticide. Elsewhere, 48 h exposure is more rapid and practical and was recommended for the standardization of this test [24].

2.3. Microscope observations

Root tips (meristem zones) were cut and placed overnight in the dark in the Carnoy fixation solution containing ethanol and glacial acetic acid (3:1) at 4 °C, and then stored in 70% ethanol. Root tips

were rinsed with distilled water and hydrolyzed with 1 N HCl for 10 min as described by Souguir et al. [18]. The root cap was removed before squashing root tissues, and samples were stained with orcein. The slides were examined under Zeiss microscope. At least three slides were stained per replica and at least 1000 cells were scored from each slide. Therefore, the analysis was conducted on an average of 9000 cells per treatment. Micronucleus frequency was calculated from the number of micronuclei scored divided by the total cells scored, and expressed in terms of Micronuclei/1000 cells. The mitotic index (MI) was determined by counting the number of mitotic cells among the total amount of scored cells (~3000) per root. Mitotic index (MI) and micronuclei (MCN) as well as aberrant mitosis frequencies were measured on the same slide.

2.4. Pigment analyses

Leaves were immersed in 80% acetone and then placed in the dark and cold. After 24 h, the sample was crushed and then filtered through gauze. The residue was taken up in acetone 80%. The filtrate was centrifuged for 5 min at 3000 rpm speed. Pellets were taken up in 80% acetone solution till removing pigments. Pigment contents then were measured by absorbance at 450, 645 and 663 nm. Concentrations of chlorophylls a and b and carotenoids were determined using equations of Mac Kinney [25] and Arnon [26]. Results were expressed as mg/g fresh weight (FW).

2.5. Statistical analysis

All experiments were repeated six times. Before performing statistical analyses, data were checked for normality and homogeneity of variance, and were square root transformed [27], with the addition of 0.5 to all data in order to avoid zeros. Statistical analyses were performed using SYSTAT 11 software for Windows with a significance level of alpha = 0.05. The Mitotic index (MI) and micronuclei (MCN) were compared using one-way ANOVA and the Dunnett multiple comparison procedure, control against treatments (10^{-5} , 10^{-4} and 2×10^{-4} M sulcotrione and HM).

3. Results

Sulcotrione treatment of *V. faba* roots for 45 h, showed root discoloration with increased doses that become darker with tannin increasing from 10^{-5} to 2×10^{-4} M sulcotrione. Different concentrations of sulcotrione (10^{-5} – 2×10^{-4} M), have reduced chlorophyll and carotenoid contents in plant leaves (Fig. 2).

Several usual pesticides (insecticides, fungicides and herbicides) were previously assayed for clastogenic and physiological activity using *V. faba* as an eukaryotic, whole-organism, test system [28]. In order to assess the genotoxic effects of sulcotrione, different concentrations were applied for a transient period of 45 h in hydroponic cultures. Mitotic index was decreasing along with sulcotrione concentration increase (Fig. 3A). Frequencies of cells with MCN in meristematic root tips plants are presented in Fig. 3B. The difference in the mean values among treatment groups are greater than would be expected by chance and there is a statistically significant difference for mitotic index ($F = 114.78$, $P < 0.001$) and for micronucleus frequency ($F = 45.150$, $P < 0.001$). Moreover multiple comparisons versus control group (Dunnett's method) shows significant differences ($P < 0.05$) between control and the four treatments.

Low micronucleus frequencies were detected in root tips of control plants. However, as expected, maleic hydrazide dramatically increased the frequency of micronuclei in root meristems as compared to control plants, showing that this herbicidal molecule can be used as a positive control for genotoxicity studies. Sulcotrione significantly enhanced the frequency of micronucleus formation in the root tips of *V. faba*.

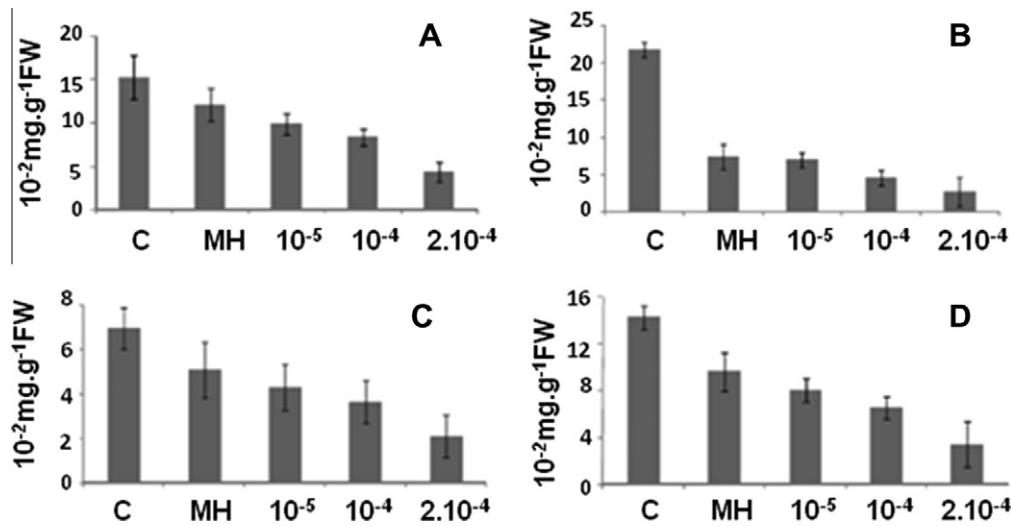


Fig. 2. Dose effect of sulcotrione (10^{-5} , 10^{-4} and 2×10^{-4} M) for 45 h at 25 °C, on pigment contents in *Vicia faba* leaves. A: Chlorophyll a contents. B: Chlorophyll b contents. C: Carotenoid contents. D: whole chlorophyll pigment contents. Values are the means of six replicas. Bars indicate SD. (c: control, MH: maleic hydrazide; 10^{-5} , 10^{-4} and 2×10^{-4} M: sulcotrione concentrations).

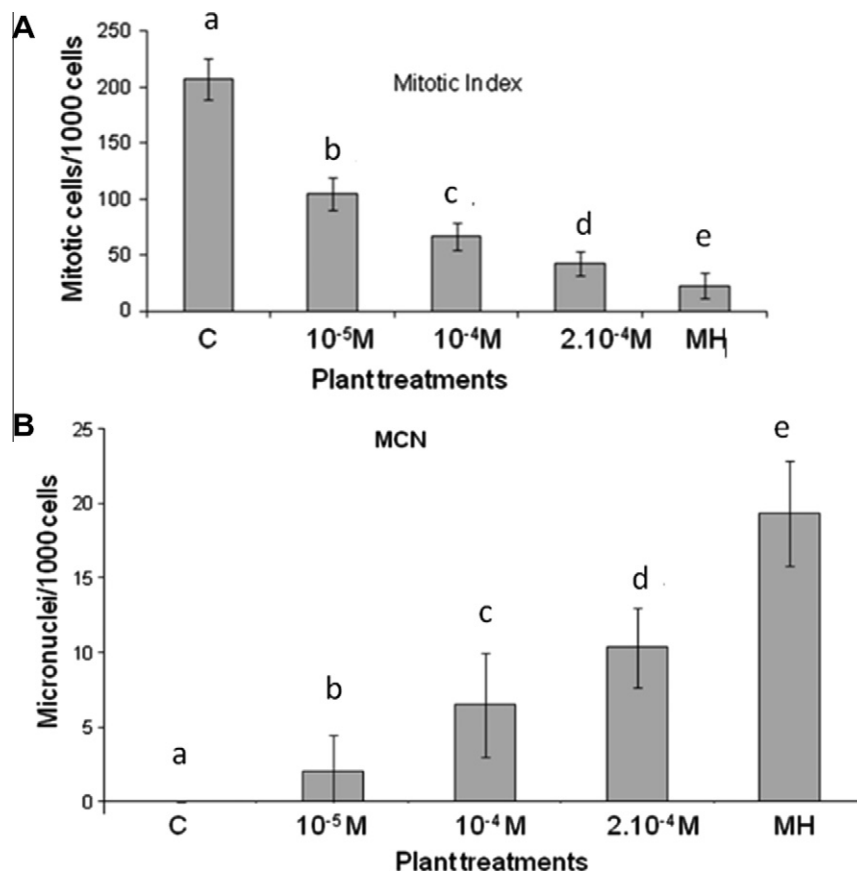


Fig. 3. Dose effect of sulcotrione on micronucleus (MCN) induction in *Vicia faba* root meristematic cells. A: Mitotic index. B: Micronuclei. (c: control untreated plants; MH: treatment by 4×10^{-3} M maleic hydrazide; treatment by different concentrations of sulcotrione, 10^{-5} , 10^{-4} and 2×10^{-4} M, for 45 h at 25 °C). Values are the means of six replicas. Bars indicate SD. Multiple comparisons versus control group (Dunnnett's method) shows significant differences ($P < 0.05$) between control and the four treatments (a, b, c, d, e, for each study).

Frequency of micronucleus formation was proportional to sulcotrione concentration added to the hydroponic solution. Micronucleus formation begins at 10^{-5} M concentration of sulcotrione. The highest frequency of cells with micronuclei was detected with a 2×10^{-4} M sulcotrione treatment. Micrographies (Fig. 4A) show presence of micronuclei, usually one in a cell, following

2×10^{-4} M sulcotrione treatments. Micronucleus (MCN) test showed that the sulcotrione was significantly genotoxic as compared to the negative control.

During the process of root cell mitosis, sulcotrione treatment allowed to show chromosomal abnormalities, mainly aneuploidies that are especially seen during anaphases (Fig. 4B). When com-

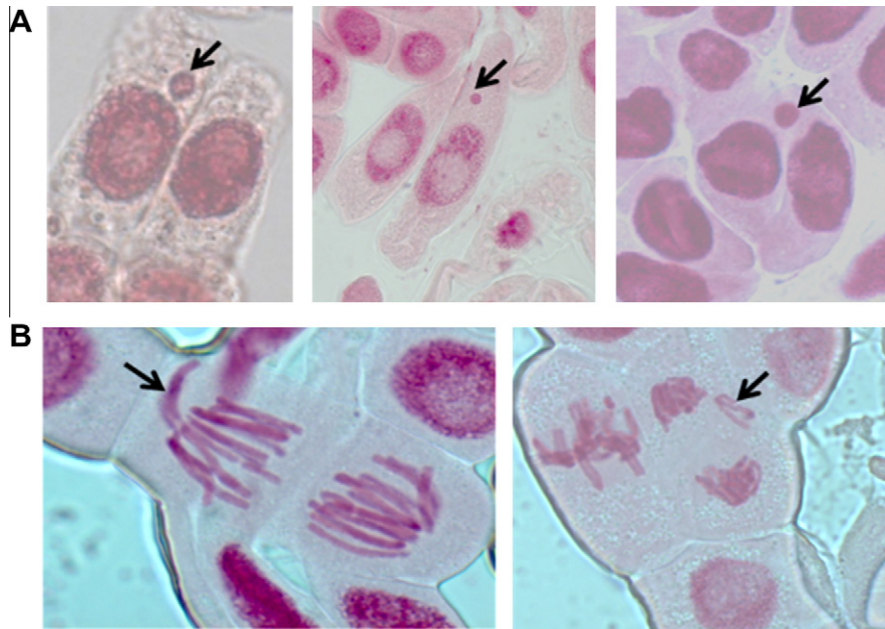


Fig. 4. Micrographies of mitosis abnormalities induced by 2×10^{-4} M sulcotrione treatment of *Vicia faba* plant meristem roots, for 45 h at 25 °C. A: arrows indicate micronuclei in interphase meristematic cells. B: arrows indicate isolate chromosomes (aneuploidy) during mitotic anaphase of meristematic cells.

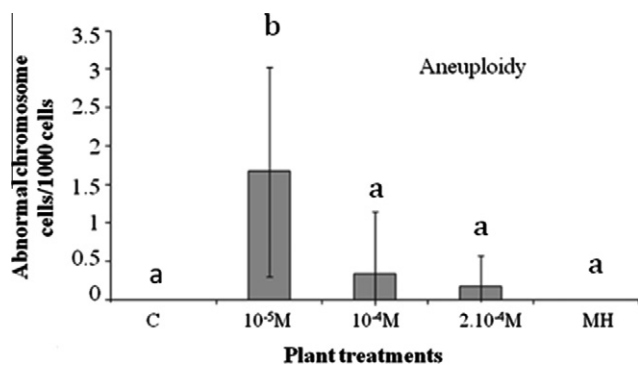


Fig. 5. Aneuploidy induction by sulcotrione treatments in *Vicia faba* root meristematic cells (c: control untreated plants; MH: treatment by 4.10^{-3} M maleic hydrazide; treatment by different concentrations of sulcotrione, 10^{-5} , 10^{-4} and 2×10^{-4} M, for 42 h at 25 °C). Values are the means of six replicas. Bars indicate SD. Multiple comparisons versus control group (Dunnett's method) shows significant differences ($P < 0.05$) between control and 10^{-5} M sulcotrione treatment but not with the three other samples (a, b).

Table 1
Percent of abnormal cells among mitotic cells.

Treatments	% Abnormal/mitotic cells
Control	0
10^{-5} M sulcotrione	1.603%
10^{-4} M sulcotrione	0.498%
2×10^{-4} M sulcotrione	0.4%
MH	0

pared to control sample, chromosomal abnormalities are significantly and mainly observed for the lowest sulcotrione concentration (10^{-5} M) and are decreased with higher concentrations. ANOVA test displayed a statistically significant difference ($F = 7.173$, $P < 0.001$) and multiple comparisons versus control group (Dunnett's method) shows significant differences ($P < 0.05$) between 10^{-5} M sulcotrione treatment and the three other treatments, a and b notations (Fig. 5). Maleic hydrazide (10^{-3} M) did

not cause any chromosomal abnormalities. The frequency of chromosomal aberrations is not correlated to mitotic index, at least for the low concentration (10^{-5} M) (Table 1).

4. Discussion

The soil is a key compartment for the fate of pesticides in the environment; a large proportion of pesticides applied in the treatment of cultures comes ground, for direct application and/or leaching of the leaves. Sulcotrione and some other triketones, as mesotrione, have a similar behavior in soils and their long half-life varies from 5 to 65 days [29] which involves hydrolysis and biotransformation processes. The persistence in soil is also related to the adsorption of active ingredients on constituents of soils. It is more important in soils rich in organic [30,31] and pH dependent [32]. Sulcotrione herbicide is applied at 250–450 g ha⁻¹ in maize post-emergence at the five to six leaf development stages. Sulcotrione is indeed moving in the soil until more than a month after application [7,9]. It is absorbed by leaves but also by roots. Water solubility of the product is 165 mg L⁻¹ at 25 °C. Wilson and Foy [31] showed that sulcotrione adsorption was correlated to the soil organic matter content, in which sulcotrione persisted only briefly, but was mobile. Cherrier et al. [7] concluded that sulcotrione had a greater potential to leach than atrazine. The presence of residues in the whole soil profile resulted from the interaction of three factors; retention, solubility and persistence [7].

The lowest amount of sulcotrione, which showed genotoxicity in bean roots, was 10^{-5} M in hydroponic conditions. For a similar culture surface, this herbicide amount represents only five times sulcotrione amounts usually spread per unit area in a field. However, it must take into account the mobility of the product in soil or in hydroponic condition, the presence of biotic and abiotic process in soil, transfer of the pesticide in aerial part of the plant to the ground, but also a possible local higher concentration of the product by sulcotrione solution runoff from different leaves of a plant. In addition, we must take into account the photo-destruction of sulcotrione which decreases the amount of herbicide available, but results in the production of inactive but toxic, stable derivative product [13,14].

Potential toxicity of sulcotrione, selective triketonic herbicide, has been earlier [10] assessed using representative environmental microorganisms frequently used in ecotoxicology [12], since this species displays a great sensitivity to pollutants. Based on the dose–response slope values *Allium* and *Vicia*, were shown as efficient test systems for root micronuclei, yet, with a greater sensitivity of *Allium* roots [33]. Previously, nine common pesticides were assayed for clastogenic and physiological activity using *V. faba* as an eukaryotic, whole-organism, test system [28].

In addition to micronuclei (MCN) formation, cytological analysis of root meristems revealed drastic changes in the organization and morphology of chromosomes (Fig. 5). Highest micronucleus value was obtained with the highest sulcotrione concentration used (2×10^{-4} M) (Fig. 3B). In contrast, chromosomal abnormalities decreased with higher sulcotrione concentrations, along with the mitotic index (Figs. 3A and 5). It was previously demonstrated relationships between chromosome abnormalities and MCN [18], but chromosome abnormalities were only seen in mitotic cells and micronuclei in interphase cells. Using another pesticide molecular type, it was shown that treatments with the insecticide dichlorvos on root meristems of *V. faba*, significantly decreased the mitotic activity and increased the frequency of chromosomal aberrations at the metaphase thus demonstrating both clastogenic and mitotic-depressive effects of DDVP on *V. faba* cells [34]. Using sulcotrione treatments, we have shown that the frequency of chromosomal aberrations is not correlated to mitotic index, at least for the lower concentration (10^{-5} M) (Table 1). The clastogenicity effect of sulcotrione treatment thus is not linked to mitosis inhibition.

Quantitation of DNA breaks was often associated with the detection of micronuclei. Some authors note similar evolution of both types of damage [35–37]; others observed an increased frequency of MCN combined with a decrease or absence of a change in the number of breaks in DNA [38,39]. The differences may be explained by the nature of induced lesions. DNA breaks quickly appear and can be repaired unlike micronuclei that require cell division, correspond to persistent lesions as non-repairable.

Disjunction between aneuploidies and micronucleus formation were described with other types of pesticides. Genotoxicity studies on organosulfite acaricide and dicarboximide fungicide have shown a lack of activity for both compounds in inducing chromosomal aberrations and SCEs. In contrast, both pesticides significantly increased micronucleus frequency [40]. The aneuploidy-inducing activity of alachlor and dichlorvos, two pesticides representing an important source of human exposure to potential carcinogens, has been evaluated in a cytokinesis block micronucleus assay combined with anti-kinetochore staining to detect chromosome loss and *in situ* hybridization with chromosome-specific centromeric probes for the analysis of non-disjunction. The two pesticides differed in their mode of action; alachlor induced both chromosomal aberrations and aneuploidy, while the genotoxic activity of dichlorvos was only related to aneuploidy induction [41]. Another pesticide, rotenone, produced DNA damage and was cytotoxic during all phases of the cell cycle. Its clastogenicity was only limited to exposures made during the G1/S and S phases [42].

Several authors have described the alteration of chromosome numbers by environmental aneuploidy-inducing agents that induce microtubule and kinetochore disorganization in mitotic cells [43,44]. A distinction to be made between micronucleus induction due to chromosomal breakage and that obtained as a result of spindle disturbances. Our data show presence of high frequencies of lagging chromosomes after exposure to the lower concentrations of pesticide. The presence of lagging chromosomes is an indication of anti-microtubule activities of the pesticides tested, as describe for other pesticides [43], but the decrease of aneuploidies quicker than mitotic index decrease as increase of micronuclei is shown, allow to do a distinction of different sulcotrione targets during cell

division (Table 1). Smaller chromosomal breakages would lead to micronucleus increase along with sulcotrione concentrations, then showing a decrease of mitotic index. Our results also corroborate the documented micronucleus formation in animal cells containing chromosomal aberrations induced by exposure to genotoxic agents [16,45].

In conclusion, this is the first report of the genotoxic effect of sulcotrione, a triketone herbicide, on plants, resulting in the appearance of micronuclei and abnormalities of mitosis.

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