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Cytotoxicity on *Allium cepa* of the two main sulcotrione photoproducts, xanthene-1,9-dione-3,4-dihydro-6-methylsulphonyl and 2-chloro-4-mesylbenzoic acid

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ABSTRACT

The cytotoxic effects of 2-chloro-4-mesylbenzoic acid (CMBA) and xanthene-1,9-dione-3,4-dihydro-6-methylsulphonyl (XDD), the two main photoproducts of sulcotrione, were investigated on *Allium* root meristematic cells at different concentrations. Degradation of sulcotrione was correlated to mitotic index decrease, together with increasing anomaly and c-mitosis frequencies. Mitotic index significantly decreased with increasing XDD and CMBA concentrations. Cell frequency with abnormal chromosomes increased with CMBA or XDD application rates. In contrast, CMBA induced a low micronucleus rate even for high concentrations while XDD increased the micronucleus ratio. C-mitoses, chromosomal aberrations due to an inactivation of the spindle, were enhanced by CMBA treatments but not by XDD. The photochemical degradation process of the pesticide can change the risk for the environment.

1. Introduction

Sulcotrione, chemically defined as 2-(2-chloro-(4-methylsulphonyl) benzoyl)-1,3-cyclohexanedione, is a triketone herbicide used in maize cultures and is proposed as an atrazine substitute [1]. It is applied on corn crop at 400 g.ha^{-1} to control development of broad leaf weeds [2]. In target plant species, sulcotrione is absorbed by leaves and roots [3,4] and inhibits the enzyme 4-hydroxyphenyl pyruvate dioxygenase (p-HPPD) [5,6], leading to a massive accumulation of phytoene and a decrease in chlorophyll and carotenoid levels [7,8], which induce necrosis and death of sensitive plants.

Sulcotrione can remain in the soil up to more than a month after application [4,9]. Sulcotrione degradation in the soil is influenced by biotic and/or abiotic factors [3]. Half-life ranges between 45 and 65 days in soil and is 8 days with microflora [2]. 1,3-Cyclohexanedione (CHD) and 2-chloro-4-mesylbenzoic acid (CMBA) are among the observed degradation compounds [10]. They arise following sulcotrione hydrolysis. Sulcotrione also absorbs solar light and undergoes direct photodegradation, which has been documented in the literature [11–13]. Photoproducts formed through irradiation of sulcotrione have been identified [11,14]. Photohydrolysis leads to CMBA and photocyclization resulting from HCl elimination yields xanthene-1,9-dione-3,4-dihydro-6-methylsulphonyl (XDD) [15]. This latter reaction is favoured upon aqueous medium acidification and when sulcotrione is irradiated as a dry deposit. These two photoproducts have no herbicide property and show toxicity towards unicellular organisms. Moreover, XDD is more toxic than sulcotrione towards *Tetrahymena pyriformis* and *Vibrio fischeri* [12,16].

Previously, we have shown that both sulcotrione and its irradiated solutions are genotoxic [17]. We have used cytogenetic bioassays on *Allium cepa* by testing the changes of the frequencies of abnormal chromosomes and mitotic index (MI). Micronucleus frequency evaluation was another way to show the potential genotoxicity of a compound. We have concluded that photodegraded sulcotrione has a greater toxicity than the parent molecule [17]. The aim of the present work was to study the toxicity of two main sulcotrione photoproducts, XDD and CMBA, by using *A. cepa* test and to compare their genotoxicity and cytotoxity.

2. Materials and methods

2.1. Material

Sulcotrione (MW = 328.77 g mol^{-1}; pKa = 3.13; boiling point 574.5 °C at 760 mmHg) was purchased from Riedel de Haën, Pestanal®, Saint-Quentin Fallavier, France. CMBA was purchased from Apollo Scientific Limited, Denton, Manchester, UK. Water was purified using a Millipore Milli-Q system (Millipore αQ, resistivity

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18 MΩ·cm, DOC < 0.1 mg.L^{-1}). Acetonitrile was a chromasolv® solvent (99%) provided by Sigma-Aldrich.

2.2. Irradiation conditions

Sulcotrione solutions (2×10^{-4} M), prepared in Milli-Q water at pH 2 directly from the standard, were irradiated in a Suntest CPS photosimulator (Atlas). A 500 W m^{-2} energy was used to simulate the sunlight average intensity received in June in France. The light intensity emitted by the xenon lamp within the wavelength range 290–420 nm was measured using a radiometer QE65000 from Ocean Optics. The internal temperature was maintained at approximately 35 ± 2 °C with cooled water (15 °C) flowing through the bottom of the sample holder with an air cooling system. Sulcotrione (200 mL) was irradiated in a 600 mL glass beaker reactor. The pH 2 was chosen for it accelerates sulcotrione photolysis. The photoproduct distribution in these conditions was close to those obtained when sulcotrione was irradiated as a dry powder on wax films mimicking the surface of leaves. After irradiation, the sulcotrione solutions were analysed by HPLC to determine the concentrations of sulcotrione, CMBA and XDD.

XDD was produced by irradiating 60 mg of sulcotrione in 100 mL of acetonitrile until complete disappearance of sulcotrione. The irradiation was conducted in a device equipped with six fluorescent tubes (TLD 15W05 Philips, Eindhoven, The Netherlands) emitting between 290 and 450 nm. The complete conversion of sulcotrione into XDD was checked by HPLC analysis. Acetonitrile was removed by evaporation using a rotavapor. XDD powder was further solubilized in ultrapure water for *A. cepa test* treatments.

HPLC-UV analyses were carried out at room temperature using a photodiode array detector chromatograph coupled with a reverse phase column (HSS T3 1.8 µm, C_{18}, 2.1 × 100 mm, Waters). A flow rate of 0.5 mL min^{-1} was used for all analyses. The gradient consisted of a mixture of 25% acetonitrile and 75% water (acidified with formic acid, pH 2.5). After 1 min, the proportion of acetone was linearly increased to 50% within 9 min. In order to monitor chromatographic separation of sulcotrione and its photoproducts, the detection wavelength was set at 284 and 243 nm. At pH 2.5, molar absorption coefficients are 5500 mol^{-1} L cm^{-1}, 27,300 mol^{-1} L cm^{-1}, and 12,000 mol^{-1} L cm^{-1} for CMBA, XDD and sulcotrione, respectively.

2.3. Plant assay system

Bulbs of onion (*Allium cepa L. var. aggregatum*) were long-half traditional shallot, 24–44 caliber class 1, Bretagne origin. Pink shallots were "Jersey" variety and were provided by organic agriculture. Root tip cells of *A. cepa* (2n = 16) were used as test system and irradiated sulcotrione, CMBA and XDD as test substances. Healthy bulbs were put in small jars of 40 mL with basal ends dipping in ultrapure water and in darkroom at room temperature (25 °C ± 2 °C). When new roots were grown to 1–2 cm length, they were washed and used. Roots of *A. cepa* were treated with irradiated sulcotrione or concentration ranges of CMBA or XDD. All chemical treatments were done in 25 mM phosphate buffer at pH 6.8 in order to neutralize solutions. Negative control was done in phosphate buffer and ethyl methanesulphonate (100 mg.L^{-1}) (Fluka) was used as positive control. Experiments were conducted for 48 h at room temperature 25 ± 1 °C in darkness.

2.4. Cytotoxicity and genotoxicity tests

Excised root tips were fixed for 24 h in Clarke's solution (ethanol 99% and glacial acetic acid 3:1) and then stored in 70% ethanol at 4 °C. Root tips were hydrolysed with 1 N HCl for 5 min and incubated in acetic-orcein (1%) for at least 30 min. Root tips were then squashed in 45% acetic-acid on slides and examined with a microscope Olympus. 3–5 bulbs were tested for each concentration of pure or irradiated treatment. 6–8 roots were examined separately for each bulb. The analysis of the mitotic index was done in random fields (≈0.2 mm^2). An average of 500 cells was scored from each different root to get a total of 3000 cells for one bulb. Each mitotic index (MI) was calculated from the number of dividing cells/total number of cells × 100. Chromosome aberrations, i.e. chromatin bridges, stickiness, stars, laggard, vagrant chromosome and fragments, were characterized in anaphase and telophase cells. All anaphase and telophase cells were taken in account in the whole meristem of each root. Only roots with at least 20 anaphases and telophases were considered for the study. Chromosomal abnormality frequency was calculated from the number of aberrant cells/Anaphase and Telophase cells ×100.

All experiments were performed in triplicate and repeated at least once. Each sample was encoded by another researcher in order to not influence the second one for determination of treatment genotoxicity.

2.5. Statistical analysis

Data from each treatment between each concentration were compared using one way ANOVA and Tukey HSD All-Pairwise Comparisons Test and correlated with JMP 10 software.

3. Results

3.1. Photodegradation sulcotrione kinetics

Photodegradation experiments were conducted with two purposes. First, we wanted to obtain irradiated solutions of sulcotrione showing different concentrations of residual sulcotrione and photoproducts. For this, sulcotrione was irradiated in acidic water for 40, 90, 150 and 210 min. The evolutions of sulcotrione, CMBA and XDD concentrations as obtained by HPLC are given in Fig. 1A. These solutions were then used in the different tests. Second, we needed to get XDD as a pure compound to test it separately. XDD could be successfully synthesized by irradiating sulcotrione in pure acetonitrile. Indeed, in these conditions, photohydrolysis of sulcotrione did not occur, therefore CMBA was not produced. Only photocyclization took place and XDD was the unique photoproduct. *A. cepa* tests were done on all these samples: irradiated solutions at different irradiation times, pure CMBA and pure XDD. HPLC analyses were done after 48 h of treatment with *A. cepa* roots to determine molecule stability. CMBA and sulcotrione are not affected by the contact with *A. cepa* and were stable all along the treatment. However, XDD was degraded during the treatment and new by-products were observed. After 48 h of treatment around 20% of XDD was degraded. Results of XDD treatment were expressed with XDD initial concentration.

Firstly, solutions obtained after different times of sulcotrione irradiation were tested. Mitotic index and anomaly frequencies were evaluated, and results are given in Fig. 1B,C. Presence of c-mitosis was also calculated in each sample (Fig. 1D). The degradation of sulcotrione was correlated to decrease of mitotic index, together with anomaly and c-mitosis frequency increases.

3.2. Mitotic index

The cytotoxic effects of CMBA and XDD compounds were investigated on *Allium* root meristematic cells at different concentrations (Fig. 2). MI was cytologically determined after 48 h treatment. Mitotic indices (MI) of *Allium* root tips incubated in ultrapure water or in 100 mg.L^{-1} ethyl methane sulphonate were 10.0% ± 0.2 and 6.8% ± 0.2 respectively. Ethyl methane sulphonate treatment, positive control,