Sulfentrazone and Flumetsulam herbicides caused DNA damage and Instability in Allium cepa test

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Abstract— *Boral 500® (sulfentrazone as active ingredient) and Scorpion® (flumetsulam as active ingredient) are herbicides widely used in Brazil´s soybean crops. U.S. Environmental Protection Agency classificated them as non-carcinogenic and no mutagenic, but literature shows that often this classification is misguided. Allium cepa assay was chosen to evaluate these herbicides, once it analyzes the frequency of micronuclei (MN), chromosomal aberrations (CA) and the mitotic index (MI). Four concentrations of each herbicide (50, 75, 100 and 125 %) were tested in triplicate using distilled water (negative control) and methyl methanesulfonate (positive control) as controls. Three experimental repetitions were realized. Boral 500® showed a higher MI in all concentrations, and higher CA and MN in the 75%, 100% and 125% concentration, with no recovery. Scorpion® showed a higher MI, CA and MN in 100% and 125% concentration, with recovery only for MI and CA. Both herbicides showed mutagenic damage and increased proliferative capacity in Allium cepa. So on, these herbicides should be revaluated as mutagenicity and carcinogenicity for responsible agencies.*

Keywords— Chromosome aberration, micronuclei, mitotic index, mutagenicity.

I. INTRODUCTION

Environmental contamination by toxic agents become matters of concern to agricultural and / or industrial economic-based countries, [(1)] once it interacts with soils and groundwater, affecting human populations and other species, with deleterious effects, such as chronical diseases development, like cancer.[(2)]

The herbicides Boral 500® (sulfentrazone as active ingredient) and Scorpion® (flumetsulam as active ingredient) are widely used in soybean crops. Recently, sulfentrazone and flumetsulam were classified as "evidence of non-carcinogenicity for humans" and "no mutagenic" by U.S. Environmental Protection Agency (EPA).[(3)] However, data about mutagenicity of these compounds are not presented in the label products, and there is only one published paper evaluating DNA damage of sulfentrazone[(4)] that showed controversial results of its classification, but none using *Allium cepa* test.

Indeed, several herbicides formulations, such as those with butachlor, atrazine and trifuralin as active ingredients, induced clastogenic and aneugenic effects assesed by *Allium cepa* test.[(5-8)] It is disturbing once *A. cepa* test showed good correlation with the results obtained in mammalians test,[(9-11)] and it were observed that this test could be more sensitive than the Ames test,[(12)] being effective to evaluate the potential risk to human health and other species.[(13-14)]

The increased frequencies of MN and CA in this assay are strong evidence of mutagenicity of the substance evaluated.[(15-17)] The mitotic index (MI) is an indicator of cell proliferation[(18)] and can be used to evaluate the level of cytotoxicity of a compound.[(7)] Furthermore, a recovery assay is necessary, once it evaluates the 'cell cycle delay', which leads to late cell responses, and even though the cells are no longer subjected to direct toxic exposure, they continue to express genotoxic or mutagenic effects.[(19,20)]

Thus, considering the lack of data and controversial information about the toxicity and the effects on DNA of Boral 500® and Scorpion®, this study aimed to evaluate the mutagenicity of such herbicides by the *A. cepa* test.

II. MATERIAL AND METHOD

2.1 The Herbicides

Boral 500® (FMC Agricultural Chemical Group, Baltimore/EUA) contains sulfentrazone (2',4'-dichloro-5-[4-difluoromethyl-4,5-dihydro-3-methyl-5-oxo-1H-1,2,4-triazol-1-yl] Metha-nesulfonanilide) as active ingredient (500 g/L), and is a member of the triazolone group. Scorpion® herbicide (Dow Chemical, Midland/EUA) has Flumetsulam (2',6'-difluoro-5-methyl [1,2,4]triazolo [1,5-a] pyrimidine-2-sulfonanilde pirimidina-2-sulfonamida) as active ingredient (120 g/L), and is part of the sulphonanilide triazolopyrimidine chemical group.

2.2 Dilution of the Herbicides

The recommended dilution for soybean cultivation depicted on the label of each herbicide was taken as 100% (Boral 500® – 1.2 Liters per cent/hectare (L.p.c/ha) and Scorpion® - 0.875 L.p.c/ha), and was further diluted in distilled water at concentrations of 50% and 75%. The concentration of 125% (Boral 500® – 1.5 L.p.c/ha and Scorpion® - 1.09 L.p.c/ha) is an extrapolation of the label for soybean crops.

2.3 *Allium cepa* assay

Seeds of *A. cepa* were germinated at room temperature (25 ± 5 °C) covered with filter paper in petri dishes. The sprouts were kept moist with distilled water until they reached 1 cm in length. After this, the filter paper was replaced and the seeds were treated (1 ml) with all dilutions of each herbicide every 8 hours, to avoid that the filter paper on the petri dishes got dry.

After 72 hours of treatment, a portion of the seedlings were fixed, while the remaining were underwent to recovery, which consisted of treatment with distilled water for an additional 48 hours, using a fresh filter paper, followed by fixation and staining procedure proposed by Grant,[10] with slight modifications. Fixation was performed using ethanol and glacial acetic acid (3:1) for 24 h. The slides were stained with Shiff´s reagent for 1 hour and then with acetic carmine. The treatments were performed in three biological replicates. Each replicate gave rise the analysis of 5.000 meristematic cells in 5 slides (1.000 cells per slide); totalizing 15.000 meristematic cells analyzed per treatment. Mitotic index (MI), micronuclei frequency (MN), and chromosomal aberrations frequency (CA) were evaluated according to Rank and Nielsen,[12] with modifications. Images were captured through an Olympus DP 71 camera connected to an Olympus BX 60 microscope, using the DP manager image software (version 3.1.1.208) (Olympus, Japan). Distilled water was used as negative control and MMS (methyl methanesulfonate, 4×10-4 M, ACROS, Geel, Belgium) was used as positive control.

2.4 Statistical Analysis

Data distribution was verified by Kolmogorov-Smirnov test. All comparisons were performed by One-Way ANOVA. When significant differences were observed (e.g. $p < 0.05$), the Dunnett's test was applied, comparing all groups against negative control. SigmaStat 3.5 (Systat Software, Inc., Chicago, Il, USA) was used to perform the statistical analyses.

III. RESULTS

MI was statistically increased after treatment in all concentrations of Boral 500® ($p< 0.05$), with no recovery. In relation to chromosome damage, were observed a statistically significant increase of CA and MN frequency for the treatments at concentrations of 75%, 100%, and 125% of Boral 500® ($p <0.05$). It indicates mutagenic damage and higher proliferative capacity. The mutagenic effect of Boral 500® was not mitigated after 48h of incubation without the herbicide (recovery protocol), indicating a long-lasting effect of this herbicide. For Scorpion® results, 100% and 125% concentrations promoted a statistically increased of MI, CA and MN frequencies ($p< 0.05$). However, after recovery, only CA and MI frequencies returned to normal rage. Additionally, significant increase for Boral 500® were observed in chromosomal losses (at concentrations of 75%, 100%, and 125%; without recovery); anaphase-telophase bridges (at concentrations of 100%, and 125%; with recovery); and chromosomal breaks (at concentration of 125%; with recovery) ($p< 0.05$) (Table 1). Significant increase for Scorpion® were observed in: anaphase-telophase bridges (at concentration of 125%), and chromosomal losses (at concentration of 75%) both with recovery ($p< 0.05$) (Table 2).

TABLE 1

MEAN AND STANDARD DEVIATION IN THE FREQUENCIES OF CHROMOSOMAL ABERRATIONS (CA), FREQUENCY OF MICRONUCLEI (MN) AND MITOTIC INDEX (MI) AND THE MAJOR TYPES OF CHROMOSOMAL ABERRATIONS FOR 5000 CELLS IN *A. CEPA* ROOTS OF BORAL 500®

Boral 500®	MI	MN	Total CA	Anaphase and Telophase Bridges	Chromosomal Breaks	Chromosomal Losses	Lagging	Others CA
Distilled Water	0.0217±0.00284	0.00046±0.00011	0.00173±0.00030	0.001±0.0002	0.00033±0.00011	0.0004±0	0±0	0±0
50%	0.0485±0.0025*	0.00066±0.00011	0.0026±0.0004	0.00146±0.00041	0.0002±0.0002	0.00086±0.00011	0±0	0.00006±0.00011
75%	0.0667±0.00541*	0.00107±0.00023*	0.00367±0.00041*	0.001733±0.00023	0.00033±0.00011	0.00133±0.00030*	0±0	0.00026±0.00023
100%	0.0699±0.00606*	0.00107±0.00023*	0.00427±0.000416*	0.002066±0.00030*	0.00046±0.00011	0.00146±0.00023*	0±0	0.00026±0.00030
125%	0.0675±0.00602*	0.00127±0.00011*	0.00527±0.00041*	0.002733±0.00030*	0.0008±0*	0.00113±0.00030*	0±0	0.00013±0.00011
MMS	0.0597±0.00061*	0.00173±0.00023*	0.0078±0.0004*	0.004466±0.00041*	0.00113±0.00030*	0.00193±0.00041*	0±0	0.00033±0.00023
Distilled Water (REC)	0.0265±0.0024	0.00033±0.00011	0.00213±0.00023	0.0012±0.0004	0.00026±0.00011	0.00066±0.00011	0±0	0±0
50% (REC)	0.0462±0.00291*	0.0008±0.0002	0.00213±0.00030	0.00126±0.00046	0.00046±0.00011	0.0002±0	0±0	0.0002±0
75% (REC)	0.0527±0.00304*	0.001±0.0002*	0.00293±0.00030*	0.00146±0.00041	0.0008±0	0.0006±0.00034*	0±0	0.00006±0.00011
100% (REC)	0.0495±0.003*	0.00107±0.00023*	0.0032±0.0004*	0.00166±0.00046	0.00073±0.00011	0.0002±0.0002*	0±0	0.00013±0.00011
125% (REC)	0.0549±0.00325*	0.001±0*	0.00353±0.00030*	0.00153±0.00011	0.00093±0.00041	0.00086±0.00023*	0±0	0.0002±0.0002
MMS (REC)	0.0586±0.00408*	0.00207±0.00041*	0.00387±0.00011*	0.00213±0.00011	0.00026±0.00023	0.00113±0.00011	0±0	0.00033±0.00030

Negative control – Distilled Water; Positive control – MMS (methyl methanesulphonate); *statistically significant differences from negative control; REC: after recovery protocol.

TABLE 2

MEAN AND STANDARD DEVIATION IN THE FREQUENCIES OF CHROMOSOMAL ABERRATIONS (CA), FREQUENCY OF MICRONUCLEI (MN) AND MITOTIC INDEX (MI) AND THE MAJOR TYPES OF CHROMOSOMAL ABERRATIONS FOR 5000 CELLS IN *A. CEPA* ROOTS OF SCORPION®

Scorpion®	MI	MN	Total CA	Anaphase and Telophase Bridges	Chromosomal Breaks	Chromosomal Losses	Lagging	Others CA
Distilled Water	0.0309±0.00693	0.00026±0.00011	0.0026±0.00052	0.00126±0.00023	0.00046±0.00011	0.0006±0.00034	0.0002±0.0002	0.00006±0.47
50%	0.0374±0.00277	0.00026±0.00011	0.003±0.00069	0.0014±0.0002	0.00006±0.00011	0.00093±0.00011	0.0006±0.0002	0±0
75%	0.0329±0.0033	0.00046±0.00046	0.00333±0.00030	0.00133±0.00023	0.0002±0.0002	0.0014±0.0002*	0.0002±0.0002	0.0002±0.0002
100%	0.0411±0.00358*	0.0008±0.0002*	0.0042±0.0002*	0.0016±0.00034	0.000333±0.00030	0.00086±0.00023	0.00106±0.00046*	0.00033±0.00011
125%	0.0417±0.00272*	0.001±0.0002*	0.00453±0.000416*	0.002±0.0002*	0.0004±0	0.001±0	0.00066±0.00030	0.00046±0.00011
MMS	0.0489±0.00197*	0.00107±0.00011*	0.0052±0.00106*	0.00206±0.00050*	0.0006±0.0002	0.00146±0.00041*	0.00073±0.00011	0.0002±0
Distilled Water (REC)	0.0298±0.00526	0.00006±0.00011	0.0018±0.00019	0.0006±0.0002	0.0000±0.00011	0.00066±0.00050	0.00026±0.00011	0.00026±0.00011
50% (REC)	0.0297±0.00304	0.00013±0.00011	0.00233±0.00041	0.00066±0.00023	0.00046±0.00011	0.00066±0.00023	0.00033±0.00011	0.0002±0
75% (REC)	0.0273±0.00319	0.00006±0.00011	0.00207±0.00030	0.0008±0	0.00053±0.00011	0.00066±0.00011	0.00006±0.00011	0±0
100% (REC)	0.0335±0.00343	0.0006±0.0002*	0.0028±0.0002	0.00126±0.00046	0.0004±0.0002	0.00066±0.00030	0±0	0.00046±0.00030
125% (REC)	0.0336±0.00851	0.00066±0.00030*	0.00253±0.00061	0.0012±0.00034	0.0002±0.0002	0.00106 ± 0.00023	0±0	0.00006±0.00011
MMS (REC)	0.0383±0.00159*	0.00066±0.00011*	0.00347±0.00090*	0.001±0.0004	0.0004±0	0.0012 ± 0.0002	0.0004±0.0002	0.00053±0.00023

Negative control – Distilled Water; Positive control – MMS (methyl methanesulphonate); *statistically significant differences from negative control; REC: after recovery protocol.

IV. DISCUSSION

In this study were used the *A. cepa* assay to detect cell cycle and chromosome alterations of two widely used herbicides for soybean crops. Many alterations were accounted as chromosomal aberrations, such as chromosomal stickness, anaphase-telophase bridges, chromosomal breaks and losses, multipolar anaphase, necrosis, nuclear buds, etc. See Figure 1.

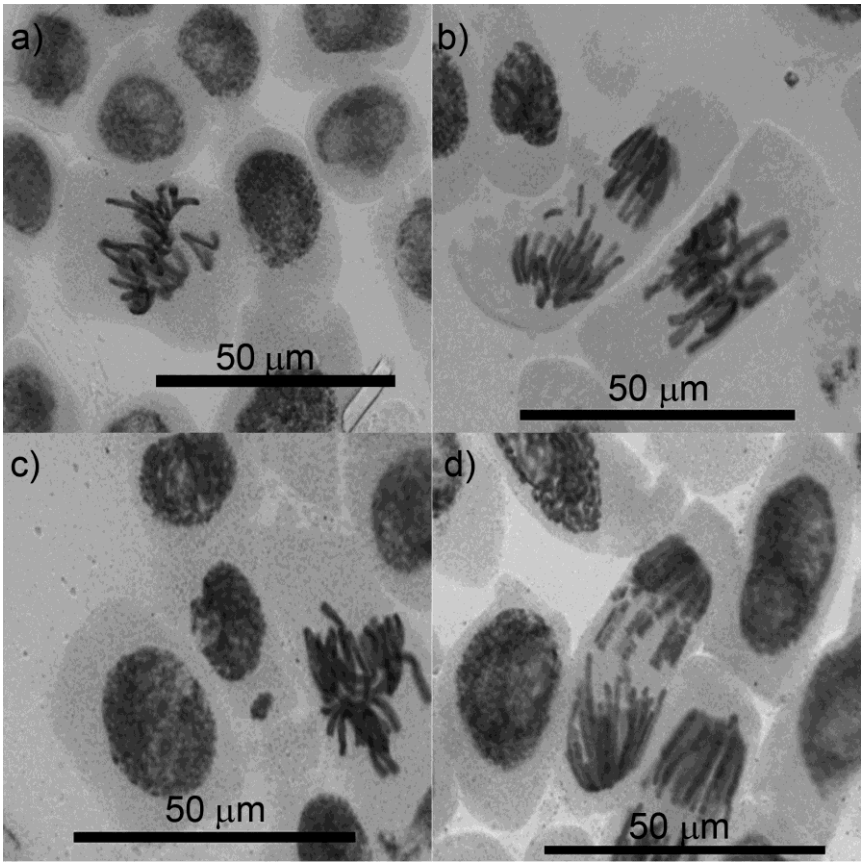

FIGURE 1: CHROMOSOMAL ABERRATIONS AND MICRONUCLEI INDUCED BY HERBICIDES IN A. CEPA ROOT SYSTEM. A) METAPHASE DELAY; B) CHROMOSOMAL BREAKS; C) MICRONUCLEI; D) BROKEN BRIDGE IN ANAPHASE.

Boral 500® herbicide is a member of the triazolone group which acts inhibiting the protoporphyrinogen oxidase in the chlorophyll biosynthesis, leading to an accumulation of intermediate toxics.(21) It is known that significant decrease of MI may be due to the mitodepressive action of the MI, indicating an alteration in the normal cell cycle and resulting in a decrease of cells in division. On the other hand, the increased MI frequency observed in all Boral 500® concentrations could be associated with abnormal cell growth[16] and / or acceleration of cell division, which, in turns, could increase the probability of chromosome damage through an error-prone cell division mechanism, as associated in cancer.(22) Indeed, at the highest concentrations of Boral 500® (75%, 100%, and 125%) were detected an increase of chromosome damage (MN and CA), even after recovery time. It indicates that this higher proliferative capacity is contributing to the elevated damage observed. Furthermore, even after 48h of no herbicide exposition, the normality was not complete, showing a long-lasting effect of this herbicide. Data about genotoxicity / mutagenicity of Boral 500® are scarce, however, Bianchi et al.(4) showed, in HepG2 cells, an increase in micronucleus rate, but no genotoxic potential, once no alteration was observed in the comet assay. In the same study was proven that a mix with another herbicide (active ingredient imidacloprid) showed an increased in the genotoxic damage in HepG2, what concerns about the use of this compounds, once they are tested separately, but used in combination in crops.

Scorpion® is an herbicide of the sulphonanilide triazolopyrimidine chemical group, whose mode of action also affects the synthesis of the Acetolactate synthase (ALS) enzyme.(23) Moreover, the ALS enzyme is not present in animals, and, in theory, the toxicity of these chemicals is specific for plants, being one of the main reasons of the use of these herbicide classes. However, our results showed that the concentrations of 100% and 125% of Scorpion® promoted a statistically significant increase of MI, CA, and MN, with partially recovery only. Moreover, Koutros et al.(13) showed a correlation between the

increase of bladder and colon cancer in farmers and the use of an herbicide whose mode of action affects the ALS enzyme; demonstrating that the deleterious effects of such herbicides is not specific for plants It only proved that the monitoring agencies must test again some herbicides to update the product label, once the literature is full of research that detected damage caused by herbicides, on an attempt to guarantee proper use and to prevent future problems.

Our study are the first which evaluated the mutagenicity of Boral 500® and Scorpion® in the *A. cepa* test, and we demonstrated a ratio among the increases in MI, and increases in MN and CA at the higher concentrations. As well as chromosome losses, anaphase-telophase bridges are statistically different in the higher (100% and 125%) concentrations of Boral 500® and (100%) of Scorpion® herbicides. it is known that anaphase bridges may be formed during an unequal exchange of chromatids or by breakage and fusion of chromosomes and chromatids, and these bridges can cause structural chromosomal mutations.(24) These data and the higher frequency of chromosomal losses and breaks probably contributed to the increased of micronuclei in both herbicides. This is worrying once micronuclei is not amenable to repair and is derived from an error due to parental cell damage,(15) such as the loss of whole chromosomes or fragments that are not incorporated into nuclei during cell division.(25)

Magdaleno et al.(8) evaluated herbicides and showed genome instability in *A. cepa* too. It shows that many herbicides have some substances in their formulation that causes DNA damage and instability, and may remain active for a long time, affecting all environment and human being. So it is important to evaluate these chemicals before use them.

Thus, these results indicate that Boral 500® and Scorpion® promoted chromosome damage and alterations of mitotic index in *A. cepa* roots. Thus, its deleterious effects should be revaluated by monitoring agencies to improve its use around the world and validate as safety for the environment and for human populations.

V. CONCLUSION

Allium cepa test showed to be an efficient test to evaluate the mutagenicity of Boral 500® and Scorpion®. Both herbicides showed mutagenic damage and increased proliferative capacity in *A. cepa*. The use of these herbicides should be revaluated and tests of mutagenicity and carcinogenicity should be made.

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