Evaluation of the genotoxic activity of dicamba and atrazine herbicides in several Mexican and South American varieties of sweetcorn (*Zea mays* L.)

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ABSTRACT. Corn is a major crop and various herbicides are used to maximize its production, which include a dicamba-atrazine mixture. This has great advantages, but can also induce DNA damage. Genotoxic activity was assessed by comet assay following application of two concentrations of dicamba-atrazine: 1000-2000 and 2000-4000 ppm. Apical meristem leaf nuclei from 119 varieties of sweetcorn plants from Mexico and South America, and from five commercial sweetcorn hybrids were used. Each accession comprised two individuals per concentration and two controls. Significant genotoxic activity ($P < 0.001$) was observed following treatment with 1000-2000 and 2000-4000 ppm compared to the negative control. There was no difference in the genotoxic activity induced by both 1000-

2000 and 2000-4000 ppm concentrations in plants from Mexico and South America ($P > 0.05$) except ($P < 0.05$) in the 2000-4000 ppm treated plants from Mexico and the 1000-2000 ppm treated plants from South America. Sweetcorn hybrids showed significant genetic damage ($P < 0.01$) at all concentrations compared to the negative controls. Thus, the dicamba-atrazine mixture caused genetic damage to corn plants, and it suggested that Mexican sweetcorn is more sensitive to dicamba-atrazine than the maize varieties from South America. Neither hybrid status nor the origin avoids DNA damage caused by Marvel. Thus, maize can be useful as a biomonitor of genetic damage induced by chemicals and to identify possible phenotypes based upon the amount of genetic damage induced by herbicides and selection of resistant genotypes.

Key words: *Zea mays* L; Dicamba; Atrazine; Genetic damage; Genotoxicity; Comet assay

INTRODUCTION

Globally, and particularly in Mexico, maize (*Zea mays* L.) is one of the most important cereals with an annual production of 22 million tonnes (FAO, 2013; SIAP-SAGARPA, 2014). This grain is used directly for feeding or for generating industrial products such as ethanol, refined oils, flours, sweets, drinks, and cosmetics (Yadav and Supriya, 2014). Various chemicals are used to increase the production and quality of crops, most notably fertilizers, insecticides, and herbicides (Bolognesi, 2003). Although herbicides offer great advantages, some produce adverse effects and may even have genotoxic activity, induce DNA damage, and affect the productivity of crops (Aksakal, 2013; Valencia-Quintana et al., 2013). Two of the most commonly used herbicides in maize crops are dicamba and atrazine, which individually or in combination, are used to control the appearance of weeds (Golla et al., 2011). Cenkci and colleagues (2010) showed that dicamba possesses mutagenic activity in legumes cells, and this herbicide also caused sister chromatid exchanges in chinese hamster ovarian cells (González et al*.,* 2009). In 2009, the Environmental Protection Agency (EPA) decided to reassess the risks to health and the environment posed by atrazine, moreover, Srivastava and Mishra (2009) reported the effect on mitotic index and an increase in the number of chromosomal aberrations in plants. In other bioassays including earthworm, quail, micro-algae, fish, rodents, human lymphocytes, HepG2 cells, and plants, the genotoxic activity of atrazine was evident (García-Villada and Reboud, 2009; Song et al., 2009; Powell et al., 2011; Campos-Pereira et al., 2012; Nwani et al., 2011).

To assess the genotoxicity of herbicides, multiple bioassays have been reported which can detect genetic damage (Álvarez et al., 2011; Valencia-Quintana et al., 2013; Zúñiga, 2013; Álvarez et al., 2014). A reliable and modern test for the detection of DNA damage is the comet test, which is highly sensitive and reproducible (Singh et al., 1988). This test detects DNA damage such as breaks in single and double stranded, covalent cross-links between DNA/DNA, DNA/protein, or base deamination by oxidative damage occurring in apurinic or apirimidinic sites, (Brendler et al., 2005; Azqueta and Collins, 2013). The comet assay has been widely used for assessing genotoxic activity of a variety of pesticides (Torres, et al., 2006; Álvarez, et al., 2014).

Dicamba-atrazine are classified as growth regulators and are used worldwide in corn crops.

In the US, many varieties of sweetcorn are severely damaged by herbicides that are applied when the young plants have emerged (Pataky and Nordby, 2006); therefore, it is important to understand the genotoxicity of the dicamba and atrazine mixture before it is placed on plants and sweetcorn from different regions of México and South America to enable informed decisions regarding their use to be made. In this study, the genotoxicity of atrazine and dicamba was assessed in apical meristem cells of sweetcorn by means of the alkaline comet test in 119 accessions from Mexico and South America.

MATERIAL AND METHODS

Plant material

A total of 119 accessions of sweetcorn varieties were evaluated, including two from México, three from South America, and five commercial hybrids from the USA: 755 Vita sweetcorn Epid'or F1, Vita 755 699, Obsession 702, Early sunglow 703, and 704 Sugar pearl Im2. The seeds were obtained from the gene bank of the Institute of Management and Use of Plant Genetic Resources (IMAREFI), University Center for Biological and Agricultural Sciences, University of Guadalajara. The material was increased in the 2012 spring-summer cycle through fraternal crosses. Seeds from each variety were sown in 60 cavities of polystyrene trays under greenhouse conditions during 2013 to obtain plants in vegetative stage 5-8.

Herbicide exposure

Individuals in vegetative stage 5-8 were subjected to two different concentrations of Marvel commercial herbicide (dicamba-atrazine) (3,6-dichloro-2-methoxy benzoic-6-chloro-N-ethyl-N-isopropyl-1,3,5-triazine-2,4-diamine): 1000-2000 and 2000-4000 ppm, which was obtained from Syngenta Agro, Mexico, DF. Two individuals per concentration and two individual controls, without application, were used for each accession. After 24-h exposure, plants were taken to the laboratory and the nuclei were isolated.

Isolation of nuclei

Nuclei from the apical meristem of plants exposed to Marvel and negative controls were obtained. Briefly, the apical cuts were washed with distilled water three times, then placed in a cold mortar with three drops of honda buffer (0.44 M sucrose, 2.5% Ficoll type 400, 5% dextran T-40, 2.5 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM mercaptoethanol, and 2.5% TritonX-100 pH 8.5). The nuclei were obtained by tissue maceration and immediately filtered through filter paper with particle retention 20-25 µm (Whatman 4).

To obtain nuclei, the filtrate was centrifuged twice at 3000 rpm (4°C) for 3 min and the supernatant was discarded. The pellet containing nuclei was resuspended in 200 µL honda buffer (Álvarez et al*.,* 2001).

Comet assay

Suspended nuclei were used in the alkaline comet assay as previously described by Koppen and Verschaeve (1996). The slide was covered with a layer of 1% Normal Melting Point

(NMP) agarose and allowed to solidify before being moved to a clean surface. Then, 300 µL 0.6% low melting point (LMP) agarose was placed on the slide. Next, 90 µL 0.5% LMP agarose was mixed with 10 µL of the nuclear suspension and the mixture was placed over the first layer. Finally, a third layer of 100 µL 0.5% LMP agarose was added to cover the second layer. Slides containing the plant nuclei were immersed in lysis solution (NaCl 2.5 mM Na_2 EDTA 10 mM, Tris-HCl 10 mM, sodium lauryl sarcosinate 1% Triton X-100 1%, and 10% DMSO, pH 10) for 24 h at 4°C. The slides were immediately washed and the gels were placed in the electrophoresis chamber (BioRad, Model A6) with cold running buffer (300 mM NaOH, 1 mM EDTA, pH 13), for 45 min at 4°C to allow denaturation and unraveling of the genetic material. Electrophoresis was carried out on the same system for 8 min at 1.0 V/cm with a current of ~300 mA. In order to avoid additional DNA damage, all of the steps described above were carried out under yellow light. Electrophoresis was performed with a Labconco power source, model 4333280. When the run-time ended, the gels were washed with distilled water for 1 min and were immediately stained with ethidium bromide for 5 min, washed twice with distilled water, and placed under a coverslip for observation. The comet assay was carried out using a fluorescence microscope (Axioskop 40) at 10X magnification with an excitation filter of 515-560 nm. The distance migrated by the DNA was measured using the Comet Assay System II Software.

Statistical analysis

DNA migration data were subjected to analysis of variance (ANOVA) using the Statistical Analysis System (SAS 9.0) and Dunnett's multiple comparison test was used to evaluate the effect of treatment of the herbicide compared to the control. A probability level of 0.05 was utilized.

RESULTS

Table 1 shows the average DNA migration distance of the nuclei from apical meristems of 119 accessions and five commercial hybrids of sweetcorn plants exposed to two different concentrations of the commercial herbicide Marvel: dicamba-atrazine 1000-2000 and dicamba-atrazine 2000-4000 ppm respectively. Significant genotoxic activity ($P \leq 0.001$) was observed when compared with the negative control. However, no significant difference ($P > 0.05$) was found between the two concentrations tested.

Table 1. Average distance of DNA migration by the nuclei of apical meristems from 119 accessions and 5 commercial hybrids of sweetcorn plants exposed to two different concentrations of the commercial herbicide Marvel: dicamba-atrazine 1000-2000 and dicamba-atrazine 2000-4000 ppm.

Populations studied	Migration average of tail length (µm)		
	Negative control	Concentration 1000-2000 ppm	Concentration 2000-4000 ppm
124	1.66 ± 0.16	8.25 ± 1.30	8.46 ± 1.48

Comparison of means between exposed and control groups $P \leq 0.001$.

Table 2 indicates the average distance of DNA migration by nuclei from apical meristems of 119 accessions of sweetcorn from Mexico and South America following exposure to two different concentrations (1000-2000 and 2000-4000 ppm) of dicamba-atrazine. There was a marked increase in tail length for the two study groups, which was greater than that of the corresponding

negative controls. In Mexican populations, greater genetic damage was observed with respect to the populations from South America, but only the 2000-4000 concentration applied in the Mexican population showed a significant difference compared to 1000-2000 ppm concentration applied in South American populations ($P < 0.05$).

Table 2. Average migration distance of DNA from the nuclei of apical meristems of 119 accessions of sweetcorn from Mexico and South America exposed to two different concentrations (1000-2000 and 2000-4000 ppm) of the dicamba-atrazine.

Populations studied	Average migration of the cauda (µm)					
	Mexican negative control	Negative control South American	Mexican concentration 1000-2000 ppm	South American concentration 1000-2000 ppm	Mexican concentration 2000-4000 ppm	South American concentration 2000-4000 ppm
119	1.61 ± 0.32	1.59 ± 0.24	8.74 ± 1.82 P < 0.01	7.12 ± 1.10 P < 0.01	8.99 ± 1.88 P < 0.01	7.61 ± 1.17 P < 0.01

Multiple comparison indicated that only the 2000-4000 Mexican and South American 1000-2000 concentrations showed significant difference of $P < 0.05$.

Figure 1 shows the average migration distance of DNA from the apical meristem of five commercial sweetcorn hybrids exposed to two different concentrations of Marvel (1000-2000 and 2000-4000 ppm). Hybrids showed significant genetic damage ($P < 0.01$) compared to the negative control. No significant difference in genetic damage was observed in hybrids between the concentrations 1000-2000 and 2000-4000 ppm.

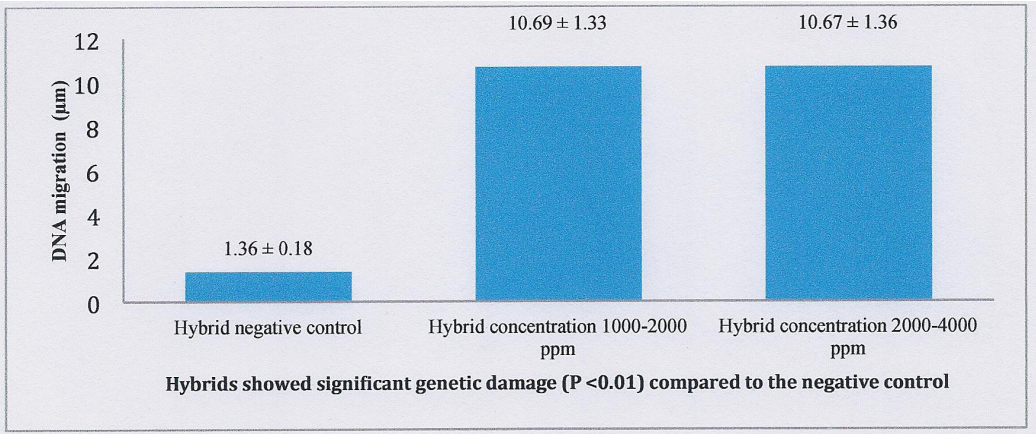

Figure 1. Average migration distance of DNA from apical meristem nuclei of five commercial hybrids of sweetcorn exposed to two different concentrations of Marvel (1000-2000 and 2000-4000 ppm).

The tail length (µm) of apical meristem nuclei from sweetcorn varieties of Mexico and South America exposed to two different concentrations of Marvel (1000-2000 and 2000-4000 ppm) and the negative control is shown in Table 3. All showed significant genetic damage ($P < 0.01$) compared to the corresponding negative controls.

Table 3. Tail length (µm) of meristematic apical nuclei of sweetcorn varieties from Mexico and South America exposed to two different concentrations of Marvel (1000-2000 and 2000-4000 ppm) and the negative control.

Origin	Populations	Negative control	Concentration 1000-2000 ppm	Concentration 2000-4000 ppm	P
Chihuahua	13	1.61 ± 0.34	10.00 ± 4.94	8.00 ± 1.54	<0.01
Durango	3	1.10 ± 0.32	9.05 ± 0.81	11.09 ± 3.87	<0.01
Guanajuato	6	1.52 ± 0.17	6.16 ± 0.54	6.32 ± 0.89	<0.01
Jalisco	18	1.81 ± 0.32	9.75 ± 2.88	9.08 ± 1.99	<0.01
Michoacán	14	1.39 ± 0.32	7.87 ± 2.33	9.26 ± 2.37	<0.01
Nayarit	2	2.04 ± 0.03	8.44 ± 3.24	8.30 ± 0.32	<0.01
Sinaloa	13	1.76 ± 0.44	8.59 ± 1.40	8.62 ± 1.79	<0.01
Sonora	15	1.50 ± 0.28	7.77 ± 2.51	8.04 ± 2.47	<0.01
Zacatecas	8	2.10 ± 0.17	8.12 ± 2.57	8.31 ± 2.49	<0.01
Argentina	10	1.90 ± 0.23	7.12 ± 1.36	7.85 ± 1.84	<0.01
Bolivia	3	1.34 ± 0.16	6.88 ± 1.17	6.49 ± 0.82	<0.01
Brazil	2	1.59 ± 0.33	5.29 ± 0.24	6.31 ± 0.07	<0.01
Chile	4	1.75 ± 0.30	7.79 ± 1.22	8.47 ± 2.57	<0.01
Ecuador	6	1.69 ± 0.20	8.34 ± 1.50	8.66 ± 2.14	<0.01
Peru	2	1.64 ± 0.26	6.85 ± 1.47	9.23 ± 1.06	<0.01

Mean and standard deviation of 92 populations from Mexico and 27 populations from South America.

DISCUSSION

Genotoxicity of the herbicide atrazine and its commercial formulation is controversial (Ribas et al., 1995; IARC, 1999; Kligerman et al., 2000; Hansen et al., 2013). Several studies in various cell types have shown that atrazine causes DNA damage (Ribas et al., 1995; Clements et al., 1997), whilst other studies do not report damage (Brusick, 1994; IARC, 1999). Moreover, at various concentrations, dicamba has also been reported to cause genetic damage (Perocco et al., 1990; Filkowski et al., 2003; Cenkci et al., 2010). Due to the controversy regarding the genotoxicity of dicamba, atrazine, and their mixture, the genotoxicity of two concentrations of Marvel (commercial mixture) was studied in: 1000-2000 and 2000-4000 ppm. Significant genotoxic activity ($P \leq 0.001$) relative to the negative control (Table 1) was clearly observed, which has been previously reported (Cenkci et al., 2010) in studies investigating the individual herbicides. It is clear that the dicamba-atrazine mixture retained and even potentiated genetic damage in corn plants. There was no significant difference ($P > 0.05$) between the two concentrations tested (Table 1), indicating that both agents are sufficiently genotoxic. Previous studies show a genotoxic effect at much lower concentrations and which is proportional to the concentration (De Campos et al., 2008; Srivastava and Mishra, 2009; Cenkci et al., 2010; Valencia-Quintana et al., 2013).

The tail length of nuclei from the apical meristem of sweetcorn from México and South America exposed to 1000-2000 and 2000-4000 ppm Marvel are presented in Table 2. There was a marked increase in tail length in both study groups that was greater than that of the corresponding negative controls. However, Mexican populations showed a slight increase in the amount of genetic damage compared with that observed in South American populations but only 2000-4000 applied in Mexican populations showed a significant difference with respect to 1000-2000 ppm in South American ($P < 0.05$). This suggests that Mexican sweetcorn are, on average, more sensitive to dicamba-atrazine than are South American maize. Pataky and Nordby (2006) demonstrated that differences in sensitivity to herbicides are due to differences in the expression of a single recessive gene, since recessive genotypes were severely damaged, whereas heterozygotes showed varying degrees of damage. Therefore, it appears that this resistance gene might be expressed to a lesser extent in Mexican sweetcorn varieties.

Some sweetcorn hybrids are damaged by post-emergent selective herbicides (Pataky and Nordby, 2006). In Figure 1 the average distance migrated by DNA from five different meristematic sweetcorn hybrids exposed to two different concentrations of Marvel (1000-2000 and 2000-4000 ppm) is shown. Regardless of the concentration used, significant genetic damage was observed compared with the negative controls ($P < 0.01$). It is clear that the hybrid status did not confer resistance to genetic damage caused by Marvel, moreover, sensitivity increased because the average tail length at both concentrations [10.69 and 10.67 µm Pataky and Nordby (2006) for 1000-2000 and 2000-4000 ppm, respectively] is markedly greater than the average length of 119 accessions studied (7.93 and 8.30 µm for 1000-2000 and 2000-4000 ppm, respectively). This is due to the uniformity of the hybrids in terms of morphological characteristics and herbicide resistance; there are morphological and physiological variables in the populations and possibly genetic variation in terms of sensitivity to herbicides, which might explain why the averages tended to be lower. No significant genetic damage was observed in the hybrids following treatment with 1000-2000 and 2000-4000 ppm concentrations, which has not been previously reported.

The means and standard deviations of sweetcorn nuclei tail length from different regions (Table 3) indicate that there was significant genetic damage ($P < 0.001$) compared to the corresponding negative controls. The origin of the sweetcorn does not confer resistance to genetic damage induced by Marvel. In all populations, damage was very high, regardless of the concentration used.

The comet assay system is efficient at evaluating genotoxicity (Gichner et al., 2003; Gichner et al., 2009; Azqueta and Collins, 2013) and is used as a biomonitor test in many organisms (Gichner et al., 2003; Olive and Banáth, 2006; Dhawan et al., 2009). Corn crops are continuously exposed to various herbicides and some are genotoxic (Perocco et al., 1990; Calvalcante et al., 2008); therefore, the use of a biomonitor to detect genetic damage in corn is relevant. Use of the comet assay in meristematic sweetcorn nuclei is an important tool for assessing the genotoxic effect of commercial herbicides, as it is fast and relatively inexpensive. Our study shows the usefulness of maize as a biomonitor of genetic damage induced by chemicals, particularly herbicides. This bioassay (comet assay) can also be applied in meristematic nuclei of normal maize; however, results regarding the genotoxicity of certain herbicides, including Marvel, could differ from those obtained in the present study with sweetcorn. This bioassay permits the genotoxicity of any type of herbicide applied to commercial maize to be assessed, and allows possible phenotypes to be identified based upon the amount of genetic damage induced by certain herbicides and subsequent selection of resistant genotypes. More studies are necessary in normal maize.

Conflicts of interest

The authors declare they have no conflict of interest.

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