at the Font Groga site. Likely, this is due to the relative high abundance of individuals belonging to the *melanogaster* group.

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Metabolic activity of diuron by *Zea mays* detected through the wing spot assay in *Drosophila melanogaster*.

Peraza-Vega, Ricardo, América N. Castañeda-Sortibrán, and Rosario Rodríguez-Arnaiz*. Laboratorio de Genética y Evolución, Departamento de Biología Celular, Facultad de Ciencias, Universidad Nacional Autónoma de México; *E-mail: rosario.rodriguez@ciencias.unam.mx

Abstract

Diuron is a phenylurea herbicide compound amply used in agriculture to control a wide variety of annual and perennial broadleaf and grass weeds, algae, and mosses. The aim of the present study was to evaluate the genotoxicity of diuron in the wing spot assay of *Drosophila melanogaster* after metabolic activation of an aqueous extract from the roots of *Zea mays* treated with different concentrations of the herbicide. Bentazone was used as positive control. The wing spot assay that assesses for somatic mutation and recombination events was carried out with standard (ST) and high-bioactivation (HB) crosses given chronic treatment to third instar larvae. Results showed that larvae treated with the aqueous extracts caused a similar positive response in both crosses.

Introduction

Diuron is an herbicide used to control a wide variety of weeds affecting maize, sugarcane, cotton, sorghum, among other crops and for fallow and idle cropland use. Diuron also has a widespread use in non-agricultural applications like industrial and rights of way uses. Due to its chemical structure, diuron has been classified as a substituted phenylurea compound. This compound can be readily taken from soil by the root system of plants and translocated into stems and leaves moving primarily via the xylem. The mechanism of action of diuron is through inhibiting photosynthesis by blocking the electron transfer at photosystem II (Wessels and van der Veen, 1956). This compound is able to bind to D-1 protein located at the reactive center of photosystem II (Arnaud *et al*., 1994; Duke, 1990).

The basic metabolism of phenylureas include N-demethylations followed by oxidation of aromatic groups (Engelhardt *et al*., 1972). In mammal cells, diuron is mainly metabolized through de-alkylation of methyl-urea groups (Abass *et al*., 2007). Diuron is capable of increasing cytochrome P450 enzymes (CYPs) activity as well as other enzymes including glutathione-S-transferase, epoxide hydrolase, and UDP-glucuronyl transferase (Schoket and Vincze, 1985, 1990). In plants, phenylureas are metabolized through N-demethylation of the nitrogen atom and hydroxylation of the aromatic group (Fonné-Pfister and Kreuz, 1990). It has been demonstrated that some enzymes belonging to CYP71 and CYP76 families are involved in diuron metabolism in plants (Fonné-Pfister and Kreuz, 1990; Höfer *et al*., 2014; Robineau *et al*., 1998; Siminszky *et al*., 1999).

Many pesticides are promutagens and become active after metabolic biotransformation by plants (Plewa, 1978). Herbicides and their metabolites represent potential health risks to humans since they are applied to food crops and may exert a genotoxic effect when they are consumed by the population. The US Environmental Protection Agency has listed diuron as a likely human carcinogen (US EPA, 1997, 2004). Also, several studies have demonstrated that diuron is a genotoxic and carcinogenic compound (Akcha *et al*.,

2012; Canna-Michaelidou and Nicolaou, 1996; Cardoso *et al*., 2010; Huovinen *et al*., 2015; Rodríguez-Arnaiz *et al*., 1989; Seiler, 1978); thus, it is important to determine the biological mechanisms involved in such processes.

In this work we wanted to determine the genotoxic properties of diuron metabolites produced by maize roots exposed to this compound by means of the wing spot assay in *Drosophila melanogaster*.

Materials and Methods

Chemical compounds

Diuron, (Koa 80WDG®) (3-(3,4-diclorofenil)-1,1-dimetilurea), Makhteshim Agan, Mexico, and Bentazon (Basagran 480 - 3-isopropil-1H-2,1,3-benzotiadiazin-4 (3H)-ona 2,2-dioxide) BASF Mexicana, were purchased from "El Sembrador" pesticide store, Mexico City.

Plant material

Maize white seeds (*Zea mays)* were obtained from "Molino de chiles y harinas El Pesado" at Santa Úrsula local market, México City.

Toxicity of Diuron

The median lethal concentration (LC_{50}) of diuron was obtained by treating third instar larvae with diuron. The herbicide was prepared at the following concentrations (mM): 31, 62.3, 125, 250, 500, and 1000. Larvae were treated in vials with the herbicide for 48 h. Three replicas were performed. With the obtained data of mortality, a polynomial regression was performed with $R^2 = 0.9739$ and a final value for the LC_{50} of 162.6 mM. However, we observed a significant increase in the duration of larval development and size reduction when larvae were exposed to concentrations above 125 mM. In order to avoid this issue, we tested 30, 60, and 120 mM concentrations.

In vivo metabolic activation of diuron by Zea mays

Five day-old primary *Zea mays* roots 3 - 5 cm long were immersed in different concentrations of diuron for 4 hours at room temperature in dark conditions. The concentrations of diuron used were 30, 60, and 120 mM. The herbicide bentazone [basagran 48%] was used as a positive control. This herbicide has been demonstrated to be genotoxic in *Drosophila* (Kaya *et al*., 2004). After treatment, the primary roots were washed with deionized water. The 3 cm tips of the main roots were homogenized at 4°C in PBS buffer, pH 7.4. The ratio of the volume of buffer solution to fresh weight of roots was 1:1 (Calderón-Segura *et al*., 2007; Takehisa *et al*., 1988). The homogenate was centrifuged at 10,000 × *g* for 15 min at 4°C. The extracted supernatant was measured for protein concentration using a Beckman spectrophotometer prior to being used in *Drosophila melanogaster* third instar larvae (Bradford, 1976).

Fly stocks and crosses

Two different stocks of flies were used, both carrying visible wing genetic markers on the third chromosome, multiple wing hairs (*mwh*, 3-0.3) and flare (*flr*^{3}, 3-38):

(1) *flr*^{3}*/In (3LR) TM3, ri p*^{p} *sep l(3)89Aa bx*^{34e} *e Bd*^{S} (*flr*^{3}/TM3, *Bd*^{S})

(2) *mwh/mwh (mwh)*

Two crosses were used, ST and HB. The ST cross was performed by mating flr^3/TM3, Bb^S females to *mwh/mwh* males. The HB cross was performed with ORR/ORR; flr^3/TM3, Bb^S females mated with *mwh/mwh* males. The ORR strain has chromosomes 1 and 2 from a Dichloro-Diphenyl-Trichloroethane (DDT)-resistant Oregon line (OR-R), which constitutively overexpresses *Cyp* genes (Graf and Van Schaik, 1992; Graf *et al*., 1984, 1989).

Wing spot assay

Eggs from both crosses were collected over 8 h into culture bottles containing a solid agar base (5%, w/v) covered with a layer of live fermenting yeast supplemented with sucrose. After 72 ± 3 h, the third instar larvae were washed from the culture bottles with a solution of 20% (w/v) sucrose and fed for the rest of the

larval development stage (approx. 48 h) with the maize aqueous extracts. For this chronic feeding, the larvae were put in vials containing 0.85 g of Drosophila Instant Medium (formula 4-24; Carolina Biological Supply, Burlington NC, USA) hydrated with the aqueous extracts. After eclosion, the adult flies were collected and stored in 70% ethanol. The wings of the transheterozygous flies from both crosses were mounted on slides and coded before scoring, at a magnification of 400×, for the presence of cell clones showing mutant wing hairs expressing *flr* or *mwh* markers (spots). The different types of spots result from different genotoxic mechanisms: point mutations, deletions, and mitotic recombination. To evaluate the genotoxic effects recorded, the frequencies of spots per wing in a treated series were compared with those of the concurrently processed negative and positive control series. These statistical comparisons were made using a computer program written by Zordan (unpublished), which uses the χ^2 test for proportions, followed by a multiple-decision procedure (Frei and Würgler, 1988, 1995). Statistical analyses were performed for single, large, twin, and total numbers of spots recovered.

Figure 1.

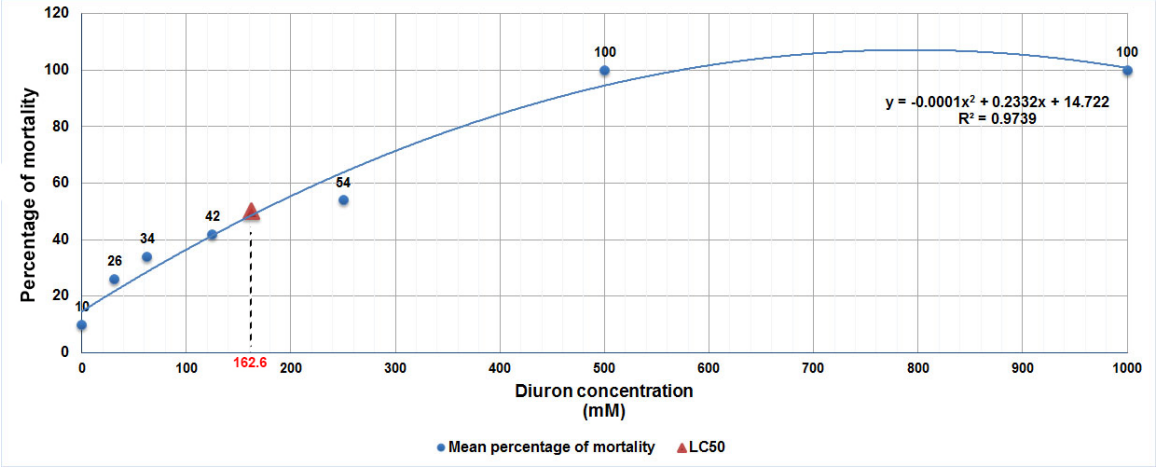
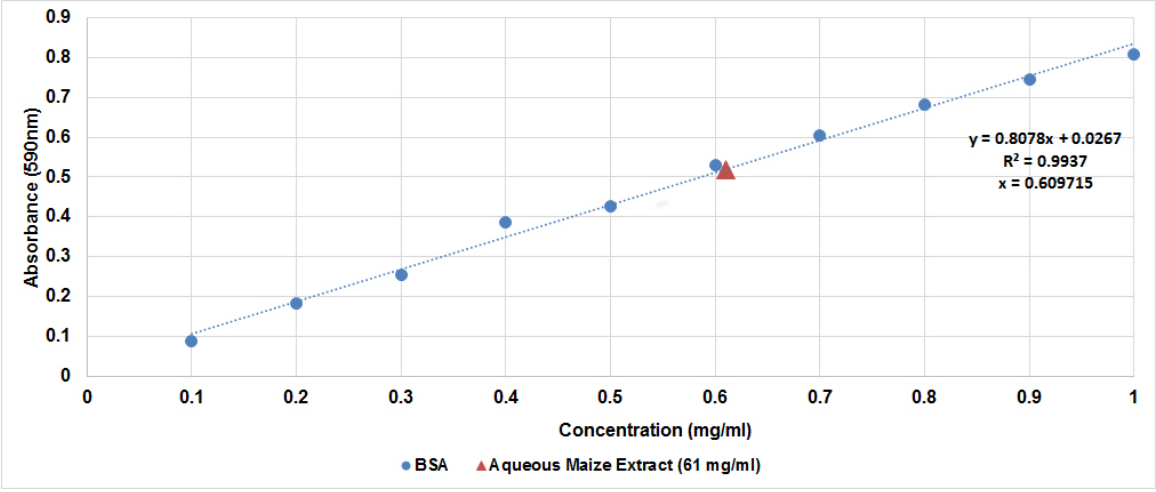


Figure 2.



Results

Figure 1 shows the percentage of toxicity induced by diuron. It can be noted that the LD_{50} was 162 mM. The protein concentration of the maize aqueous extracts in the three replicas assayed was almost constant at 61 mg/ml (Figure 2). A summary of the results obtained in the ST and HB progeny after treatment with the maize aqueous extract and the positive bentazone control is shown in Table 1. Notably, the induction of all types of spots is very similar for both crosses, the majority being small single spots. The positive control

bentazone also gave similar results independent of the type of cross. Comparison of the induced effects between both types of treatment shows that the treatment of larvae with the maize extract reduces the number and frequency of small single and total spots at the highest diuron concentration in the ST cross, but not in the HB cross. Despite this reduction, a positive effect in total spot frequency at all concentrations used was found. Thus our study shows that chronic treatment of third instar larvae produced similar dose-response effects in both crosses.

Table 1. Summary of results obtained in trans-heterozygous progeny of ST and HB crosses after chronic treatment of larvae with the maize extract treated with Diuron.

		Spots per fly (Number of spots) Statistical diagnoses[b]					
Concentration (mM)	Cross [a] and Number of flies	Small single spots (1-2 cells) m = 2	Large single spots (>2 cells) m = 5	Twin spots m = 5	Total Spots m = 2	Spots with mwh clone	Mean number of cell division cycles
	ST						
Negative Control	60	0.35 (21)	0 (0)	0 (0)	0.35 (21)	21	1.48
Bentazone	60	0.43 (26)i	0.12 (7)+	0.05 (3)i	0.60 (36)+	36	1.89
30	60	0.52 (31)i	0.08 (5)+	0.05 (3)i	0.65 (39)+	39	1.74
60	60	0.57 (34)i	0.08 (5)+	0.07 (4)i	0.72 (43)+	43	1.93
120	60	0.55 (33)i	0.05 (3)i	0.03 (2)i	0.63 (38)+	38	1.76
	HB						
Negative Control	60	0.28 (17)	0.05 (3)	0 (0)	0.33 (20)	20	1.65
Bentazone	60	0.62 (37)+	0.05 (3)i	0.03 (2)i	0.70 (42)+	42	1.62
30	60	0.50 (30)+	0.20 (12)+	0.03 (2)i	0.73 (44)+	43	2.23
60	60	0.73 (44)+	0.07 (4)i	0.02 (1)i	0.82 (49)+	49	1.73
120	60	0.72 (43)+	0.10 (6)i	0.02(1)i	0.83 (50)+	50	1.76

[a] ST standard cross; HB high bioactivation cross. [b] Statistical diagnoses according to Frei and Würgler (1988; 1995), m: minimal risk multiplication factor for the assessment of negative results. For the final statistical diagnoses of all outcomes: + = positive; - = negative; = inconclusive and w+= weakly positive with the standard SMART software based in the conditional binomial test according to Kastenbaum-Bowman significance levels (a = b = 0.05) (Frei and Würgler,1988). The non-parametric Mann-Whitney Wilcoxon U-test with significance levels (a = b = 0.05; one-sided) was used in order to exclude false positive or and negative diagnoses (Frei and Würgler, 1995).

Discussion

To evaluate the genotoxicity of diuron comparatively, the larvae of ST and HB crosses were directly treated with the maize aqueous extract of the herbicide. The HB cross is characterized by a higher sensitivity to progenotoxins, because the ORR *flr*^{3}/TM3, *Bd*^{S} strain carries chromosomes 1 and 2 from a DDT-resistant Oregon R-(R) line, which has an increased level of CYPs (Graf and Van Schaik, 1992). The majority of spots recovered after treatment were single spots of one or two cells, which could be due to induced genotoxicity at a late stage of development of the wing imaginal discs due to delayed metabolism. In this study, twin spots were not significantly produced at the concentrations assayed. In mammals diuron is able to induce CYP expression of the isoform CYP3A4 (Abass *et al*., 2012).

Herbicide metabolism in plants is largely due to the biotransformation of substrate by CYP450 enzymes. These compounds are absorbed by the roots and accumulate in the meristem. Soybean CYP71A10

catalyses the metabolism of phenylurea herbicides, thus converting the herbicides of this class to more polar compounds. This enzyme functions as a N-demethylase for diuron (Siminszky *et al*., 1999). In *Arabidopsis thaliana*, the CYP76C subfamily is involved in both natural and xenobiotic metabolism, particularly for phenylurea herbicide biotransformation (Höfer *et al*., 2014). In *Zea mays*, a CYP71C family cluster of four CYP genes was first described by Frey and collaborators (Frey *et al*., 1995). The genes are located on the tip of the short arm of chromosome 4 and are involved in biosynthetic pathways of secondary metabolites (Dutartre *et al*., 2012). Because plants do not have an excretion system, we were interested in studying the effects of maize metabolism on the capability of CYPs from *Zea mays* to modulate genotoxicity to diuron, particularly because plants accumulate secondary metabolites from herbicides. Our results showed a similar dose-response effect in both crosses, hence, aqueous extracts of the roots of *Zea mays* caused similar results in both crosses. Thus, no differences were observed between crosses after extract treatment, a result that probably is due to a lack of post-biotransformation in larvae and suggests that the biotransformation of the secondary metabolites is probably due to the CYP71C family of maize. Although plants and animals have phase I and II enzymes (Parkinson and Ogilvie, 2008), conjugated metabolites are generally excreted in animals, but are stored in plants (Sandermann, 1988, 1992). These important differences could be related to the results obtained in this study. Accumulation of the secondary metabolites in maize roots produced a similar effect in both fly crosses due to both having received the same secondary metabolites produced by the biotransformation of diuron in maize. This effect could be related to the metabolism of other reported pesticides (Abass *et al*., 2007, 2010, 2012; Buratti *et al*., 2003; Croom *et al*., 2010; Foxenberg *et al*., 2007; Leoni *et al*., 2008; Mutch and Williams, 2006; Poet *et al*., 2003; Usmani *et al*., 2004).

The positive control bentazon was genotoxic in both crosses and showed a similar trend. Furthermore, maize metabolism did not modulate the biotransformation of bentazon. Our results are quite different from those reported in 2008 by Heres-Pulido and collaborators using wheat extracts. We found similar induction of total spots, independent of the cross that was used (ST 0.60 *vs.* HB 0.70), whereas they found significant differences (ST 0.82 *vs*. HB 1.62).

Conclusions

Maize aqueous extract was shown to be genotoxic at every concentration used for both crosses in the wing spot assay. No dose-response effect was observed. These results indicate that maize roots are able to biotransform diuron and produce metabolites capable of causing the same level of genotoxic effects in the ST and HB crosses of *Drosophila melanogaster*.

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Drosophila species associated with fresh chili peppers.

Oliveira, Berenice Tristão[1], Vera Lúcia da Silva Valente[1,2,3], and Rebeca Zanini[1,2].
[1]Laboratório de *Drosophila*, Universidade Federal do Rio Grande do Sul (UFRGS), Avenida Bento Gonçalves, 9500 prédio 43323 sala 210 CEP 91501-970, Bairro Agronomia, Porto Alegre, RS, Brazil; [2]Programa de Pós-Graduação em Biologia Animal, UFRGS, Avenida Bento Gonçalves, 9500 prédio 43435 CEP 91501-970, Bairro Agronomia, Porto Alegre, RS, Brazil; [3]Programa de Pós-Graduação em Genética e Biologia Molecular, UFRGS, Avenida Bento Gonçalves, 9500 prédio 43323M, Bairro Agronomia, Porto Alegre, RS, Brazil.

Capsicum baccatum var. *Pendulum* (Willd.) Eshbaugh (The plant list 2016) (Solanaceae) is popularly known as "lady's finger chili" or Cambuci chili, in Brazil, or Ají, in Peru. This species is one of the five species belonging to this genus (Eshbaugh, 1968; 1970) and is native to South America, being found in lowlands of Argentina, Bolivia, Brazil, Chile, Colombia, Ecuador, and Peru (Jarrett, 2007).

In June of 2016 some Drosophilidae were observed in a sample of *C. baccatum* from a farm located in Alvorada, Rio Grande do Sul, Brazil (30° 0' 1" S, 51° 4' 42" W). This farm has a chili plantation and swine facilities.

The fruits were collected and placed in 500 mL vials containing vermiculite and plugged with synthetic foam stoppers (2 fruits per vial). The vials were kept in an incubator with constant temperature (21±1ºC), humidity (60% rh), and photoperiod (12h:12h). Emerged adults were transferred to vials with