

# EXHIBIT 120



Research Article

## Comparison of the *in vivo* and *in vitro* genotoxicity of glyphosate isopropylamine salt in three different organisms

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### Abstract

There is considerable controversy with regard to the genotoxicity of glyphosate, with some reports stating that this compound is non-toxic for fish, birds and mammals. In this work, we used the comet assay to examine the genotoxicity of glyphosate isopropylamine (0.7, 7, 70 and 700  $\mu\text{M}$ ) in human lymphocytes, erythrocytes of *Oreochromis niloticus* and staminal nuclei of *Tradescantia* (4430) *in vitro* and *in vivo*. Cells, nuclei and fish that had and had not been exposed to 5 mM N-nitrosodiethylamine (NDEA) were used as positive and negative controls, respectively. Significant ( $p < 0.01$ ) genetic damage was observed *in vivo* and *in vitro* in all cell types and organisms tested. Human lymphocytes and *Tradescantia* hairs showed lower genetic damage *in vivo* compared to *in vitro*, possibly because of efficient metabolism of the herbicide. In *O. niloticus* erythrocytes, significant ( $p < 0.001$ ) genotoxicity was observed at  $0.7 \mu\text{M}$ , whereas *in vitro*, glyphosate was genotoxic in human lymphocytes and *Tradescantia* hairs at  $0.7 \mu\text{M}$ . These results indicate that glyphosate is genotoxic in the cells and organisms studied at concentrations of 0.7-7  $\mu\text{M}$ .

**Key words:** comet assay, DNA damage, genotoxicity, glyphosate.

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### Introduction

Glyphosate (N-(phosphonomethyl) glycine) is usually produced as glyphosate isopropylamine salt, one of the most widely used herbicides (Cox, 1998). The US Environmental Protection Agency (EPA) classified glyphosate as category E, indicating “evidence of no carcinogenicity for humans” (EPA, 1993) and, according to the US Forest Service (1997), glyphosate has no adverse effects in humans. Glyphosphate is also reportedly not genotoxic in soil microorganisms (Owczarek *et al.*, 1999; Busse *et al.*, 2001; Conner and Black, 2004; De Roos *et al.*, 2005; Dimitrov *et al.*, 2006). Chronic feeding studies of glyphosate have provided no evidence of a carcinogenic effect in mice or rats (Williams *et al.*, 2000). The risk of genotoxicity in humans is low in areas where glyphosate is applied for coca and poppy eradication (Bolognesi *et al.*, 2009), but an associa-

tion with multiple myeloma has been suggested (De Roos *et al.*, 2005). In contrast, several studies using different tests have reported high genotoxicity for glyphosate (Sivikova and Dianovsky, 2006; Sparling *et al.*, 2006; Cavas and Könen, 2007; Alvarez *et al.*, 2011; Guilherme *et al.*, 2012), with the genotoxicity observed being related to the test system used (Zúñiga, 2001), *e.g.*, plants (Dimitrov *et al.*, 2006; Alvarez *et al.*, 2011; Truta *et al.*, 2011), fish (Cavas and Könen, 2007) and human cells (De Roos *et al.*, 2005; Bolognesi *et al.*, 2009) are reportedly very sensitive to glyphosate.

Comparisons between the cells of different organisms *in vivo* and *in vitro* using the comet assay system can be important in assessing the genotoxicity of glyphosate. The comet assay system, which was first used in human lymphocytes, is very efficient in detecting genotoxicity (Singh *et al.*, 1988) because it allows the visualization of damage directly in the genetic material of individual cells. This test has also been used in plants and fish (Koppen and Verschaeye, 1996; Alvarez *et al.*, 2001; Guilherme *et al.*, 2012).

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In this study, the genotoxicity of glyphosate was assessed by applying the comet assay to *Tradescantia* (clone 4430) staminal nuclei, tilapia (*Oreochromis niloticus*) erythrocytes and human lymphocytes. The findings in the latter cells were compared with the genetic damage in lymphocytes from workers occupationally exposed to glyphosate based on data from a previous report (Paz-y-Miño *et al.*, 2007).

## Materials and Methods

### Chemicals

Glyphosate (N-(phosphonomethyl)-glycine), 96% (CAS No. 1071-83-6, lot 09816 PE) was obtained from Aldrich Chemical Co. (St. Louis, MO, USA) (Cox, 1995). The concentrations tested were 0.7, 7, 70 and 700  $\mu\text{M}$ , with 5 mM N-nitrosodiethylamine (NDEA, CAS No. 55-18-5) being used as a positive control. These concentrations were used in a previous investigation (Alvarez *et al.*, 2011).

### Organisms studied

For each concentration, eight tilapia (*O. niloticus*) and 30 *Tradescantia* plants were studied, in addition to the corresponding negative and positive controls, *in vivo* and *in vitro*. In the case of human lymphocytes *in vitro*, cells from eight individuals were also used. Paz-y-Miño *et al.* (2007) studied the direct effect of glyphosate in occupationally exposed humans. Their study was done in similar conditions to ours with respect to the control groups. Blood samples were tested using the alkaline comet assay as described (Singh *et al.*, 1988; Paz-y-Miño *et al.*, 2007). Comets were analyzed on a Zeiss fluorescence microscope fitted with a 50 W mercury lamp and an excitation filter of 515-560 nm. While the above cited authors used a calibrated ocular micrometer, we used a comet assay program. Nonetheless, the use of either method did not affect the final tail length measurements because the comparison was in microns.

### Preparation of cells and nuclei

For the preparation of human lymphocytes, peripheral blood samples were obtained from a finger puncture in young students who initially responded to a questionnaire to rule out exposure to genotoxic agents. Individuals on medical treatment, smokers, drug users and inhabitants of the contaminated area of Guadalajara, Jalisco, Mexico were excluded from the study. Each blood sample was placed in a test tube containing 3 mL of phosphate-buffered saline (PBS; 160 mM NaCl, 8 mM  $\text{Na}_2\text{HPO}_4$ , 4 mM  $\text{NaH}_2\text{PO}_4$  and 50 mM EDTA; pH 7) and immediately centrifuged at 3000 rpm for 5 min. The supernatant was removed and the pellet was resuspended in phosphate buffer and immediately stored at 4 °C until used.

Tilapia erythrocytes were from blood collected from specimens  $10 \pm 3$  cm in length obtained from Banco Nacional Genómico de Tilapia *Oreochromis niloticus*. The fish

were acclimatized in 5000 L aquaria under a natural photoperiod in aerated, recirculating tap water, with the following physicochemical conditions: salinity 0, temperature  $20 \pm 1$  °C, pH  $7.3 \pm 0.2$  and dissolved oxygen  $8.1 \pm 0.5$  mg/L. During this period, the fish were fed with fish roe every other day. The experiment was done in 20 L aquaria, in static mode. The fish were deprived of food for one day before the experiment and during the experimental period. Thirty-two fish were divided into four aquaria (groups of eight fish per treatment) and exposed to different concentrations of Roundup® (Monsanto; Roundup is the trademark name for a glyphosphate product) (*in vivo* treatment). Another two aquaria with clean water served as a negative control and positive control (5 mM NDEA). Each glyphosate concentration was tested during a 20 h exposure and there was no mortality at any of the concentrations. After each exposure, 0.5 mL of erythrocytes was obtained by branchial puncture and the cells then washed and centrifuged in ice-cold PBS, as mentioned for human lymphocytes.

Cell viability was assessed with the Trypan Blue test using 20 mL of peripheral blood lymphocytes and 20 mL of circulating erythrocyte suspension. The mean percentage viability for each group was > 89%.

*Tradescantia* (clone 4430, a *T. subacaulis* X *T. hirsutiflora* hybrid that is highly sensitive to environmental mutagens) was used to prepare stamen nuclei. The plants were grown under controlled conditions, at a daytime temperature of 22 °C and night-time temperature of 16-18 °C. For each concentration, 30 inflorescences (~10 flowers containing 1500-3000 stamen hairs) were immersed for 3 h in 250 mL of the desired concentration of glyphosate isopropylamine salt (*in vivo* study). The same number of inflorescences and time interval were used for the negative and positive controls (Hoagland's solution and 5 mM NDEA, respectively). The experiment was done in duplicate. After treatment, the inflorescences were washed with distilled water and placed in Hoagland's solution. On day 6 after treatment (the point at which the mutation became evident (Underbrink *et al.*, 1973), indicating that it had not been repaired), the stamen nuclei from treated plants were used for the comet assay and were separated according to Alvarez *et al.* (2001).

For the *in vitro* study, stamen nuclei from ten flowers of untreated plants for each experimental condition were placed in a cold mortar with 500  $\mu\text{L}$  of Honda buffer (0.44 M sucrose, 2.5% Ficoll (type 400), 5% Dextran T-40, 25 mM Tris-HCl (pH 8.5), 10 mM  $\text{MgCl}_2$ , 10 mM  $\beta$ -mercaptoethanol and 2.5% Triton X-100) and homogenized for 2 min, after which the mixture was filtered through a nylon mesh (80  $\mu\text{m}$ ). The nuclei were separated by centrifugation (3000 rpm, 4 °C, 5 min) and washed three times in 5 mL of wash solution (0.4 M sucrose, 50 mM Tris base and 5 mM  $\text{MgCl}_2$ , pH 8.5), resuspended in 200  $\mu\text{L}$  of the same solu-

tion and stored at  $-20^{\circ}\text{C}$  until electrophoresis (Alvarez *et al.*, 2001).

Slides of *Tradescantia* nuclei, fish erythrocytes and human lymphocytes were prepared according to Singh *et al.* (1988). Slides with nuclei from untreated plants were exposed to each concentration of glyphosate isopropylamine salt for 3 h at  $25^{\circ}\text{C}$ , washed three times with distilled water and then stored at  $4^{\circ}\text{C}$  to prevent repair of the induced damage. Other slides with nuclei from cells that had or had not been exposed to 5 mM NDEA were used as positive and negative controls, respectively. Slides with stamen nuclei from treated plants (*in vivo* experiment) were also prepared.

Slides of human lymphocytes and fish erythrocytes from the *in vitro* experiments were exposed to each concentration of glyphosate isopropylamine salt for 20 h at  $25^{\circ}\text{C}$ , washed and then stored at  $4^{\circ}\text{C}$ , as described above. Slides of cells that had or had not been exposed to NDEA were used as positive and negative controls, respectively. Cells from fish that had or had not been exposed to 5 mM NDEA were used as positive and negative controls, respectively, in the *in vivo* experiments. The entire experiment was repeated twice.

### Comet assay

The nuclei from *Tradescantia* stamens and human lymphocytes were used in the comet assay, as described by Singh *et al.* (1988) and Alvarez *et al.* (2001). The slides were immersed in lysis buffer (2.5 M NaCl, 100 mM  $\text{Na}_2\text{EDTA}$ , 10 mM Tris-HCl, 1% sodium lauryl sarcosine, 1% Triton X-100 and 10% DMSO, pH 10) for 20 h at  $4^{\circ}\text{C}$  to ensure nuclear lysis and then placed in a horizontal electrophoresis system with a high pH buffer (30 mM NaOH, 1 mM  $\text{Na}_2\text{EDTA}$ , pH 13) for 45 min to allow DNA unfolding before electrophoresis for 20 min (*Tradescantia* nuclei and human lymphocytes) or 10 min (fish erythrocytes) at 1.0 V/cm with an amperage of  $\sim 300$  mA. The same electrophoretic unit and power supply were used throughout the study (Hartmann *et al.*, 2003). All of the steps described above were done under yellow light to prevent additional changes to the DNA.

After electrophoresis, the slides were gently washed to remove the alkaline solution and then immersed in neutralization buffer (0.4 M Tris base, pH 7.5) for 5 min. The gels were stained with ethidium bromide (100  $\mu\text{L}$  at 20  $\mu\text{g}/\text{mL}$ ) for 3 min and then rinsed three times with distilled water. The preparation was subsequently covered with a coverslip and the slides were examined by fluorescence microscopy using a light microscope equipped with a 515-560 nm excitation filter. Nuclei and cells were observed at 10X magnification and tail length during migration was determined by using Comet assay software based on published protocols (Hartmann *et al.*, 2003). Approximately 50 cells or nuclei per slide and two slides for each experimental point and controls were evaluated.

### Data analysis

The results were expressed as the mean  $\pm$  SD and were analyzed by one-way analysis of variance (ANOVA) using the CoStat program (Ma *et al.*, 1994). All experimental groups were compared with the corresponding negative control using the Dunnett test. Fifty cells were used for all organisms. For *Tradescantia*, the number of hairs/nuclei tested in the treatments varied from 50 to 250, depending on flower availability. A value of  $p \leq 0.05$  indicated significance.

## Results

### Comet assay of human lymphocytes

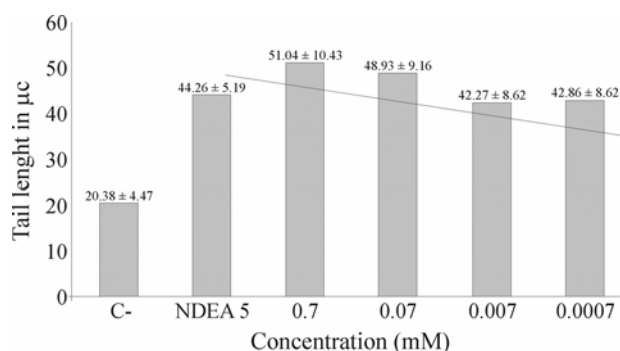
Figure 1 shows that the migration (tail length) of human lymphocyte DNA in the comet assay increased with the concentration of glyphosate and was generally proportional to the latter. The responses to different concentrations of glyphosate differed significantly among themselves ( $p < 0.0001$ , ANOVA) and were significantly different from the negative and positive controls ( $p \leq 0.01$ , Dunnett test).

### Comet assay of *O. niloticus* erythrocytes

Figure 2 shows the comet assay results for tilapia erythrocytes exposed to different concentrations of glyphosate isopropylamine salt *in vitro* and *in vivo*. When tested *in vitro*, the increase in DNA migration was proportional to the glyphosate concentration ( $p \leq 0.001$ ), although no genotoxicity was observed at 0.0007 mM. The responses to different concentrations of glyphosate differed significantly among themselves ( $p < 0.0001$ , ANOVA) and were significantly different from the negative and positive controls ( $p \leq 0.01$ , Dunnett test). Glyphosate was also genotoxic to fish erythrocytes *in vivo* ( $p \leq 0.001$ ), but the response was not concentration-dependent.

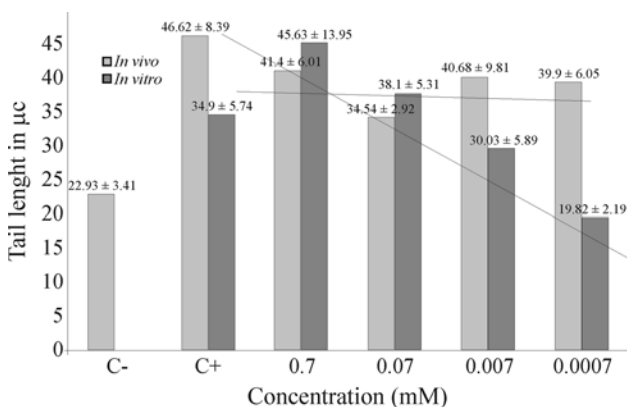
### Comet assay of *Tradescantia* stamen nuclei

The comet assay results for *Tradescantia* stamen nuclei exposed to glyphosate *in vitro* and *in vivo* are shown in

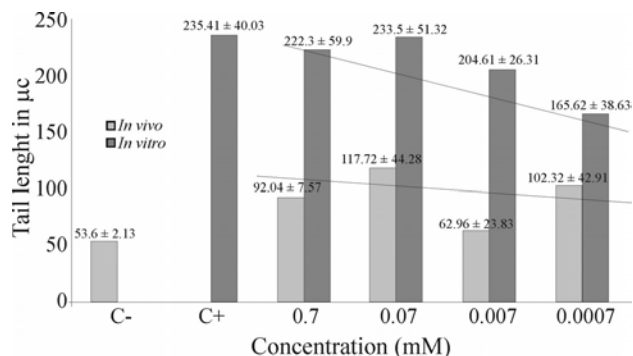


**Figure 1** - Tail length in human lymphocytes exposed to different concentrations of isopropylamine glyphosate. The diagonal line indicates the relationship between glyphosate concentration and tail length in micrometers ( $\mu\text{m}$ ). NDEA5 - 5 mM N-nitrosodiethylamine. Negative control (C-). The values above the columns are the mean  $\pm$  SD ( $n = 8$ ). Tail length  $\mu\text{m}$ .





**Figure 2** - Tail lengths of *O. niloticus* erythrocytes exposed to different concentrations of isopropylamine glyphosate *in vitro* and *in vivo*. The diagonal line and horizontal line indicate the relationship between glyphosate concentration and tail length in the *in vivo* and *in vitro* studies, respectively. C- and C+ - negative and positive controls, respectively. The values above the columns are the mean ± SD (n = 8). Tail length (µc).



**Figure 3** - Tail lengths of stamen nuclei from *Tradescantia* inflorescences exposed to different concentrations of isopropylamine glyphosate *in vitro* and *in vivo*. The lines indicate the relationship between glyphosate concentration and tail length in the *in vivo* and *in vitro* studies. C- and C+ - negative and positive controls, respectively. The values above the columns are the mean ± SD (n = 30). Tail length (µc).

Figure 3. *In vitro*, there was a positive relationship between the glyphosate concentration and the increase in DNA migration (at glyphosate concentrations of 0.0007 to 0.07 mM); there were also significant differences (p :: 0.0001) among the responses to these three concentrations of glyphosate, and between all glyphosate concentrations and the negative control (p :: 0.01). Although glyphosate was also significantly (p :: 0.05) genotoxic *in vivo*, this response was not proportional to the concentration tested and was significantly lower than that observed *in vitro*.

Table 1 compares the genotoxicity of glyphosate in human lymphocytes, *O. niloticus* erythrocytes and *Tradescantia* (clone 4430) stamen nuclei *in vivo* and *in vitro*. Glyphosate was clearly genotoxic in all cases.

**Discussion**

The comet assay is a valuable and sensitive tool for detecting genetic damage in individual cells (Singh *et al.*, 1988). Alvarez *et al.* (2001) reported a protocol that simplifies this assay in *Tradescantia* stamen nuclei. Genetic damage induced by glyphosate has been reported (Mañas *et al.*, 2009; Vera-Candiotti *et al.*, 2013) and the comet assay has been used to demonstrate genotoxicity in fish hepatic cells and *Tradescantia* nuclei (Alvarez *et al.*, 2011; Guilherme *et al.*, 2012). Although the evidence of glyphosate genotoxicity or carcinogenicity from *in vitro* and animal studies is

poor (De Roos *et al.*, 2005), a few epidemiological reports have indicated potential health effects (De Roos *et al.*, 2003).

In this study, the comet assay clearly showed that glyphosate was genotoxic in the cells examined. Since the *in vivo* effect of glyphosate in humans was not examined in this work, we compared the data from a study of the direct effect of glyphosate in occupationally exposed humans (Paz-y-Miño *et al.*, 2007) with the effect of direct exposure of human lymphocytes to glyphosate observed here. As shown in Table 2, glyphosate was genotoxic in both studies. The tail length was very similar in both cases and there was a positive relationship between genotoxicity and glyphosate concentration/dose.

The high genotoxicity of glyphosate in human lymphocytes, *Tradescantia* nuclei and fish erythrocytes *in vitro* may be indicative of direct DNA damage (Kirkland, 1998; Torstenson *et al.*, 1989; Alvarez *et al.*, 2011). The genotoxicity of glyphosate was lower *in vivo* than *in vitro*, perhaps because of efficient herbicide degradation in the former situation. This generalization did not apply to erythrocytes of *O. niloticus* since these fish were particularly sensitive to glyphosate, perhaps because of poor herbicide metabolism; this finding could also explain the lack of concentration-dependent genetic damage. Glyphosate has previously been reported to cause DNA damage in liver cells of *Anguilla anguilla* (Guilherme *et al.*, 2012) but differently, *Oreochromis niloticus* erythrocytes showed to be more susceptible to lower concentration.

**Table 1** - Comparison of the genotoxicity of glyphosate in human lymphocytes *in vitro* and *O. niloticus* erythrocytes and *Tradescantia* stamen nuclei (clone 4430) *in vivo* and *in vitro*.

Human cells		<i>O. niloticus</i> blood cells		<i>Tradescantia</i> (clone 4430) stamen nuclei	
<i>In vitro</i> exposed cells	<i>In vivo</i> exposed fishes	<i>In vitro</i> exposed cells	<i>In vivo</i> exposed <i>Tradescantia</i> plants	<i>In vitro</i> exposed nuclei	
PE	PE	PE	PE	PE	PE

PE - positive effect.

**Table 2** - Comparison between human lymphocytes from persons occupationally exposed to glyphosate (Paz-y-Miño *et al.*, 2007) and human lymphocytes exposed directly to various concentrations of the compound.

Study <i>in vivo</i> (Paz-y-Miño <i>et al.</i> , 2007)	mM	Tail length (µc)
Individuals exposed		35.5 ± 6.4
Individuals not exposed		25.9 ± 0.6
Study <i>in vitro</i> (present study)		
Lymphocytes exposed	0.7	51.0 ± 10.4
	0.07	48.9 ± 9.2
	0.007	42.3 ± 8.6
	0.0007	42.9 ± 8.6
Lymphocytes not exposed		20.4 ± 4.1

The values are the mean ± SD (n = 8).

Rank *et al.* (1993) investigated the potential genotoxicity of glyphosate in *A. cepa*. The anaphase-telophase *Allium* test showed that glyphosate significantly increased the genetic damage at concentrations of 1.44 mg/mL and 2.88 mg/mL. Our results suggest that the minimal concentration for glyphosate genotoxicity was close to 0.0007 mM (118 µg/L), although genotoxicity may start at even lower concentrations. This finding agrees with other studies that have tested similar concentrations, *e.g.*, 58-116 µg/L (Alvarez *et al.*, 2011; Guilherme *et al.*, 2012). However, high concentrations have been reported by Kültigin *et al.* (2011) (100-500 mg/L) and Prasad *et al.* (2009) (25-50 mg/L).

The selection of an adequate bioassay to detect genotoxicity is a very important factor in obtaining useful results (Zúñiga, 2001). In the present study, the comet assay was clearly sufficiently sensitive to detect the genotoxicity of glyphosate isopropylamine in cells and nuclei of different organisms.

In conclusion, our results indicate that glyphosate is genotoxic, depending on the time and concentration used, as reported by Poletta *et al.* (2009). Given the extensive use of this herbicide, it is clear that glyphosate has a potential risk for a variety of organisms, including humans.

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