



Evaluation of genetic damage induced by glyphosate isopropylamine salt using *Tradescantia* bioassays

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Abstract

Glyphosate is noted for being non-toxic in fishes, birds and mammals (including humans). Nevertheless, the degree of genotoxicity is seriously controversial. In this work, various concentrations of a glyphosate isopropylamine salt were tested using two methods of genotoxicity assaying, viz., the pink mutation assay with *Tradescantia* (4430) and the comet assay with nuclei from staminal cells of the same plant. Staminal nuclei were studied in two different forms, namely nuclei from exposed plants, and nuclei exposed directly. Using the pink mutation assay, isopropylamine induced a total or partial loss of color in staminal cells, a fundamental criterion utilized in this test. Consequently, its use is not recommended when studying genotoxicity with agents that produce pallid staminal cells. The comet assay system detected statistically significant ($p < 0.01$) genotoxic activity by isopropylamine, when compared to the negative control in both the nuclei of treated plants and directly treated nuclei, but only the treated nuclei showed a dose-dependent increase. Average migration in the nuclei of treated plants increased, when compared to that in treated nuclei. This was probably due, either to the permanence of isopropylamine in inflorescences, or to the presence of secondary metabolites. In conclusion, isopropylamine possesses strong genotoxic activity, but its detection can vary depending on the test systems used.

Key words: glyphosate, DNA damage, comet assay, plant genotoxicity test, *Tradescantia* point mutation test.

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Glyphosate (N-[phosphonomethyl] glycine), usually in the form of a glyphosate isopropylamine salt, is one of the most widely used herbicides in the world, but serious contradictions exist regarding its genotoxicity. According to the U.S. Forest Service (1997), glyphosate does not show adverse effects on soil microorganisms, and the absence of genotoxic activity has also been reported by others (Owczarek *et al.*, 1999; Busse *et al.*, 2001; Conner and Black, 2004; De Roous *et al.*, 2005; Dimitrov *et al.*, 2006). Nevertheless, several works have reported high genotoxic activity by glyphosate (Li and Long, 1988; Bolognesi *et al.*, 1997; Clements *et al.*, 1997; Peluso *et al.*, 1998; Kaya *et al.*, 2000; Piesova, 2005; Sivikova and Dianovsky, 2006; Sparling *et al.*, 2006; Cavas and Könen, 2007; Grisolia, 2002; Ramirez and Cuenca, 2002) and although this herbicide has been studied using different bioassays, the results are not

conclusive, and apparently depend much on the system used (Zúñiga, 2001).

One of the most commonly used bioassays, also considered extremely reliable, for evaluating genotoxicity is the pink mutation assay with *Tradescantia* staminal hairs. This test is highly valued for its simplicity, and it is used to detect the effect of a wide spectrum of chemical agents and complex mixtures (Underbrink *et al.*, 1973; Schairer *et al.*, 1982; Ahmed and Grant, 1992; Grant and Salamone, 1994; Ma *et al.*, 1994; Sandhu *et al.*, 1994). In this assay, color changes in cells from floral parts are used to determine mutational events. The heterozygote condition (Aa) is used, wherein blue colour is expressed as dominant, while pink is recessive. The loss, inactivation or mutation of the dominant allele produces a pink cell (Underbrink *et al.*, 1973).

Another sensitive test for detecting genotoxic activity, the comet assay, facilitates direct visualization of damage in genetic material from individual cells (Singh *et al.*, 1988). This test, already used with *Vicia faba* (Koppen and

Verschaeve, 1996), has also proved to be efficient with nuclei of *Tradescantia* (Alvarez-Moya *et al.*, 2001).

Although the toxicity of glyphosate in plants has already been completely defined, specific genotoxic activity itself has been less studied and is less well-known. Thus, studies on plants can be very important to clarify its genotoxicity. In this work, the pink mutation test with *Tradescantia* staminal hairs and the comet assay system with staminal hair nuclei from the same plant were realized. The latter was applied to exposed nuclei and the nuclei of exposed plants.

N-(phosphonomethyl)-glycine 96% (CAS No. 1071-83-6, lot 09816 PE) was obtained from Aldrich. The evaluated concentrations were 0.7, 0.07, 0.007 and 0.0007 mM. In order to find the appropriate concentration range, a preliminary test was carried out, in which *Tradescantia* plants were exposed 10 by 10 to decreasing concentrations of that used in commercial cultures (70 mM being the approximate concentration that causes death of the target plant). The initial concentration not inducing plant-death was 0.7 mM.

The *Tradescantia*, clone (4430) (hybrid *T. subacaulis* X *T. hirsutiflora*), which is highly sensitive to environmental mutagens, was used here. The plants were grown under controlled conditions, with a daytime temperature of 22 °C and night-time temperatures ranging from 16 to 18 °C. For each concentration, 30 inflorescences were immersed for 3 h in 250 mL of the isopropylamine concentration to be tested, which signifies around 15 flowers (1500 to 3000 stamen hairs) to be assayed daily in each experimental group. The same number of inflorescences and time-span were used with both positive (nitrosodiethylamine (NDEA) 1 mM) and negative (Hoagland's solution) controls. The experiment was carried out in duplicate. Subsequently, the inflorescences were washed with distilled water and placed in Hoagland's solution until the moment of reading.

A part of the inflorescences was used for pink-mutation counting, whereas nuclei extracted from the other part were employed in comet assaying. The stamen hairs in each experimental point were observed daily from the 7th to 14th day after application of isopropylamine, a period during which the pink mutations become clearly visible. The mutational events, as well as the number of observed hairs and cells, were scored under a dissecting microscope. The frequency of pink cells (events per 1000 cells) was established according to the methodology described by Underbrink *et al.* (1973). The mean values of pink mutations are shown with 95% confidence intervals ($p < 0.05$).

Staminal hair-cell nuclei from treated plants were separated according to Alvarez-Moya *et al.* (2001). The stamens of ten flowers from treated plants for each experimental point, obtained on the 6th day after treatment, when mutation is becoming evident, thus indicating the absence of repair (Underbrink *et al.*, 1973), were then placed into a cold mortar with 500 μ 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 MgCl₂, 10 mM β -mercaptoethanol, and 2.5% Triton X-100), to then be homogenized for 2 min. Subsequently, the mixture was filtered through a nylon mesh (80 μ m). The nuclei were separated by centrifugation at 999 g (4 °C) for 3 min. Under these conditions, gradient centrifugation and freezing in liquid nitrogen to break the cell wall were not necessary for separating nuclei. These were then washed three times in 5 mL of a wash solution (sucrose 0.4 M, Tris-Base 50 mM and MgCl₂, pH 8.5), re-suspended in 200 μ L of the same solution, and then stored at -20 °C until electrophoresis. Slides were prepared according to Singh *et al.* (1988).

The nuclei of untreated plants were used in the comet assay, according to Singh *et al.* (1988) and Alvarez-Moya *et al.* (2001). Stamens of ten flowers were used for each concentration of glyphosate isopropylamine salt and respective controls. The same methodology was employed to obtain the previously mentioned nuclei. Slides with nuclei were then exposed for 3 h at 25 °C to each concentration of glyphosate isopropylamine salt to be probed, washed 3 times with distilled water, and then kept at 4 °C to avoid the repair of the induced damage. The experiment was repeated twice.

To induce nuclear lysis and facilitate DNA unfolding, all the slides were immersed in a lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 1% sodium lauryl sarcosine, 1% Triton X-100, and 10% DMSO, pH 10) for 1 h at 4 °C. These were then placed in a horizontal electrophoresis system with a high pH buffer (30 mM NaOH, 1 mM Na₂EDTA, pH 13) for 45 min to allow unwinding of DNA prior to electrophoresis which was carried out for 15 min at 1.0 V/cm with an accompanying amperage of approximately 200 mA. The same electrophoresis unit and power supply were used throughout the study (Hartmann *et al.*, 2003). In order to avoid additional DNA damage, all the steps described above were carried out under yellow light.

Following electrophoresis, the slides, first gently washed to remove alkali, were then immersed in a neutralization buffer (0.4 M Tris-Base, pH 7.5) for 5 min. Gels were stained with ethidium bromide (100 μ L at 20 μ g/mL) for 3 min and then rinsed three times with distilled water. The preparation was then coverslipped. Fluorescence microscopy used to examine the slides employed a light microscope equipped with a 515-560 nm excitation filter. Nuclei were observed at 40X magnification, and migration determined by the visual scoring of tail length, according to published protocols (Hartmann *et al.*, 2003). Approximately 50 nuclei per slide and two slides for each experimental point and controls, were evaluated.

The data obtained were submitted to one-way analysis of variance testing (ANOVA) using the Co Stat program (Ma *et al.*, 1994). The Dunnett test was used for comparing the negative control with data from the nuclei of exposed plants and the exposed nuclei of healthy ones. Each experiment was repeated twice. Depending on flower-

availability, the number of hairs proved to be different in each treatment. Results were considered statistically significant at $p = 0.05$.

The pink mutation assay is highly efficient for evaluating genotoxicity (Underbrink, 1973; Grant and Salamone, 1994; Ma *et al.*, 1994). Nevertheless, it was not possible to obtain the average of pink mutation frequency from the 7th to 14th day after treatment in glyphosate isopropylamine salt, since all the concentrations (0.07-0.0007 mM) induced partial or total loss of color in staminal cells, this affecting the fundamental criterion for scoring genotoxic activity of a chemical compound, *viz.*, the change in color from blue (Aa) to pink (aa).

The pink mutation assay in *Tradescantia* turned out to be inadequate for detecting isopropylamine mutagenic activity, and most certainly for other chemical agents potentially capable of clarifying staminal cells. Possibly, the use of inadequate bioassays for detecting genotoxicity in this herbicide, has generated contradictory information, as has also occurred in other cases (Zúñiga, 2001). Although the pink mutation assay was not a good option for studying the genotoxic activity of glyphosate, which was not discarded in *Tradescantia*, we performed the comet assay system on the nuclei of the same plant and results were very different.

The comet assay is a valuable and sensitive tool for detecting genetic damage in individual cells (Singh *et al.*, 1988). According to Alvarez-Moya *et al.* (2001), there is also a protocol that simplifies this assay in *Tradescantia* staminal nuclei. A significant difference ($p < 0.01$) was evidenced when comparing controls and the different isopro-

pylamine concentrations in both treated plants and treated nuclei (Figure 1).

Furthermore, beginning at 0.07 mM, there was also evidence of an increase in dose-dependence, probably due to the direct contact of isopropylamine with nuclear DNA. These results are compatible with several other reports (Ramirez and Cuenca, 2002; Grisolia, 2002; Cavas and Konen, 2007). Moreover, there was an increase in mean migration in the nuclei of exposed plants, when compared to exposed nuclei.

We believe that there are two plausible explanations for this behavior, namely (1) the formation of a secondary mutagenic metabolite of isopropylamine in the *Tradescantia* plants but not in the nuclei (Torstensson *et al.*, 1989), and (2) the longer exposure time in assayed nuclei from treated plants. It is worthy of note that, although the treatment was administered for 3 h and the inflorescences washed extensively to eliminate residues, glyphosate (or its possible secondary metabolite) present within the plant for 6 days could increase genetic damage. This also might explain why a clear relationship between dose and response was not observed. Based on these results, we believe that isopropylamine used in commercial farming can induce genetic damage, depending on the dose used and the physiological characteristics of the plants exposed to it.

The selection of a bioassay to detect genotoxicity is very important for obtaining confiable results. As was observed for the pink mutation test, isopropylamine interferes with evaluation criteria, thereby generating incorrect results. The comet assay system clearly detected the genotoxicity of isopropylamine, but the magnitude varied depending on the type of exposure.

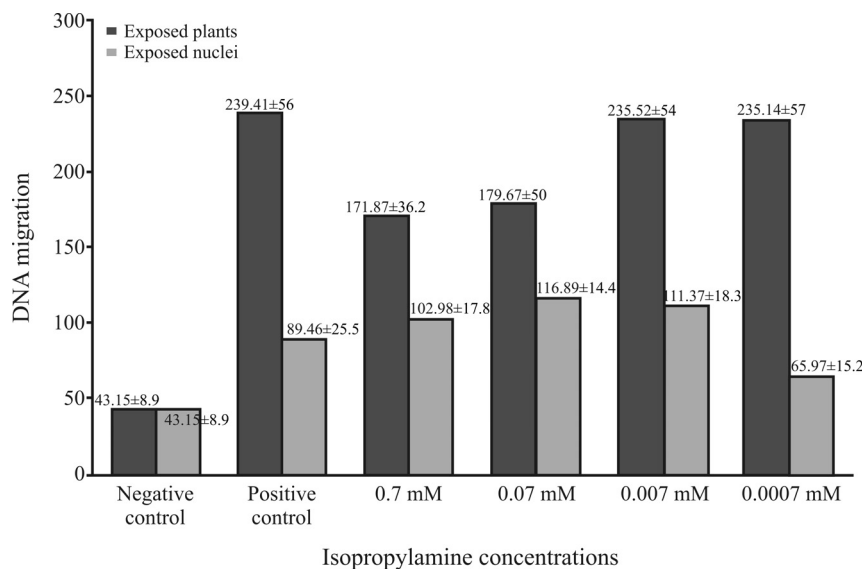


Figure 1 - Average tail length and standard deviation in μm of *Tradescantia* staminal hair nuclei of both plants and nuclei exposed to different concentrations of isopropylamine salt of glyphosate and to two controls, negative (Hoaglands solution) and positive (EMS 1 mM). The mean tail length of the negative control in both exposed plants and nuclei was significantly different ($p < 0.05$), when compared with the positive control and each glyphosate concentration. Data are an average of the experiments run in duplicate.

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