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Glyphosate-based herbicides modulate oxidative stress response in the nematode *Caenorhabditis elegans*

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ABSTRACT

Glyphosate-based formulation is used as non-selective and post-emergent herbicides in urban and rural activities. In view of its recurring applications in agricultural producing countries, the increase of glyphosate concentration in the environment stresses the need to test the adverse effects on non-target organisms and assess the risk of its use. This paper analyzes the toxicological and oxidative stress and modulatory effects of a glyphosate commercial formulation (glyphosate F) on the nematode *Caenorhabditis elegans*. We detected ROS production and enhancement of oxidative stress response in glyphosate F-treated nematodes. Particularly, we found an increased *ctl-1* catalase gene expression of a catalase specific activity. In addition, we showed that glyphosate F treatment activated the FOXO transcription factor DAF-16, a critical target of the insulin/IGF-1 signaling pathway, which modulates the transcription of a broad range of genes involved in stress resistance, reproductive development, dauer formation, and longevity. In summary, the exposure of glyphosate F induces an oxidative imbalance in *C. elegans that leads* to the DAF-16 activation and consequently to the expression of genes that boost the antioxidant defense system. In this regard, *clt-1* gene and catalase activity proved to be excellent biomarkers to develop more sensitive protocols to assess the environmental risk of glyphosate use.

1. Introduction

Glyphosate is one of the most widely used agrochemicals around the world as a result of the expansion and increased production of genetically modified crops (Coupe et al., 2012; ISAAA, 2016; US Environmental Protection Agency, 2016). It is a non-selective broad-spectrum herbicide developed for the removal of grasses, sedges, herbs and woody shrubs, especially perennials. Due to precipitation, runoff and leaching processes, glyphosate and its metabolites can be found as environmental contaminants in soil and waters, enhancing the risk of negative impacts on non-target organisms and further deteriorating ecosystem health (Peruzzo et al., 2008; Struger et al., 2015; Yang et al., 2015).

Glyphosate is a glycine analogue that harms plants by suppressing their capacity to generate aromatic amino acids (Boocock and Coggins, 1983). The potential environmental and health risk associated with their applications has been the topic of scientific and social discussion since it was first used in 1970 (Mesnage et al., 2015). Glyphosate salts alone have low to very low toxicity to mammals, but in commercial formulations, such as Roundup® or TouchDown®, they have proved to be more toxic due to the addition of surfactants (for a review see Mesnage et al., 2015). Indeed, the penetration of active ingredient through the cell membrane and subsequent intracellular action is greatly facilitated by these adjuvants (Haefs et al., 2002; Marc et al., 2002). Since the active ingredient is never used alone, further assessments are needed to determine the negative impact of the herbicide formulations on the ecosystem. Given their several advantages, toxicological experiments using the nematode *Caenorhabditis elegans* could be an excellent alternative to address this concern.

C. elegans is a free-living nematode, one of the most abundant phyla

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in nature, which plays an important role in ecosystem services. Due to its ubiquitous appearance and ecological relevance, this nematode is a suitable indicator to assess environmental pollution (Clavijo et al., 2017, 2016; Frézal and Félix, 2015; Leung et al., 2008; Tejeda-Benitez et al., 2016). Another interesting aspect is that many of the *C. elegans* physiological processes and stress responses are conserved in higher eukaryotes (e.g., humans) (Hunt, 2017). Therefore, the increasing amount of evidence supports *C. elegans* as a valuable toxicity model to predict outcomes in higher organisms.

Toxicological studies are greatly simplified in this nematode compared to more traditional animal models because it is a simple and small organism that has a short lifespan (4 days at 20 °C) and produces hundreds of offspring in each generation (Brenner, 1974). Additionally, powerful molecular tools have been developed for *C. elegans* that provide researchers with the means to probe the mechanisms underlying toxicity (Kaletta and Hengartner, 2006). In view of the above, it can be stated that *C. elegans* is a successful animal model for environmental mechanistic toxicology research.

To date, only a few reports have analyzed the adverse effects of glyphosate-based herbicides on *C. elegans*, but the mechanisms were not fully characterized (Ruan et al., 2009). It was demonstrated that exposure to TouchDown® during the first and second larval stages (L1 and L2) causes neuronal degeneration, particularly of dopaminergic neurons (Negga et al., 2012, 2011). McVey et al. (2016) also showed that this product affected the nervous system development of *C. elegans* embryos. Therefore, investigating the effects of glyphosate-based herbicide on nematodes may elucidate defense mechanisms and toxicological effects for this species to predict the herbicide effects on higher organisms.

Environmental pollutants, such as pesticides, induce a cellular disturbance through an increase in reactive oxygen species (ROS), commonly referred to as oxidative stress, that often precedes the onset of long term effects such as the impairment of the immune response and reproduction capacity, premature aging and a lower survival rate (Miranda-Vizuete and Veal, 2017; Monserrat et al., 2007). Several molecular mechanisms are capable of detoxifying ROS and regulating the redox balance of the cell. The enzyme superoxide dismutase degrades superoxide anion into peroxide which is then decomposed into water by the enzyme catalase and/or peroxiredoxin. These major systems act in conjunction with the thioredoxin system, composed of the thioredoxin protein and the thioredoxin reductase enzyme, and with the glutathione systems, that include glutathione, glutathione reductase, glutathione peroxidases, glutaredoxin and glutathione-S-transferases. As regards *C. elegans*, more than eighty genes are involved in the antioxidant defense systems. They include three catalase encoding genes (*ctl-1* to *ctl-3*), five superoxide dismutase encoding genes (*sod-1* to *sod-5*), 44 glutathione-S-transferase genes (*gst-1* to *gst-44*), one glutathione reductase gene (*gsr-1*), eight glutathione peroxidase genes (*gpx-1* to *gpx-8*), two thioredoxin reductase genes (*trxr-1* and *trxr-2*), three peroxiredoxins genes (*prdx-2*, *prdx-3* and *prdx-6*) (Back et al., 2012).

Hence, the aim of this paper is to analyze the toxicological and oxidative stress modulatory effects of glyphosate-based herbicides on the nematode *C. elegans*. First, we established the consequences that the glyphosate commercial formulation (glyphosate F) has on the generation of ROS. As antioxidant defense mechanisms are biomarkers widely used to assess toxic stress, we focused our attention on exploring the oxidative stress modulatory effects on acute exposure of glyphosate F. We detected ROS production and enhancement of oxidative stress response in glyphosate F-treated nematodes. Particularly, we found a pronounced increase in the expression of *ctl-1* gene and catalase specific activity. Finally, we tested if glyphosate F treatment could activate the FOXO transcription factor DAF-16, the major downstream target of the *C. elegans* insulin/IGF-1 signaling pathway, which modulates the transcription of a broad range of genes involved in stress response, reproductive development, dauer formation, and longevity (Murphy, 2006).

2. Materials and methods

2.1. Chemicals

Commercial formulations of glyphosate (GlifosatoAtanor II®, distributed by Atanor, Munro, Buenos Aires province, Argentina), containing monopotassium salt of *N*-phosphonomethyl glycine at 43.8 g L^{-1} as the active ingredient, and of paraquat (Gramoxone Super®, distributed by Syngenta Chemicals B.V., Belgium), containing paraquat dichloride at 276 g L^{-1}, were used. The Sigma-Aldrich Chemical Company supplied 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) and Roche and Trizol Reagent from Invitrogen supplied the complete mini protease mix. All other chemicals were of the highest purity available.

A stock solution of commercial herbicide with M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, 1 mL of 1 M $MgSO_4$, and H_2O to 1 L, pH 7.2) was freshly made. Different volumes of the stock solution were mixed with M9 buffer to achieve working concentrations expressed in terms of the final glyphosate concentration presented in each assay. The initial glyphosate concentration in stock solution was analytically determined at LABFAUBA (Facultad de Agronomía, Universidad de Buenos Aires) by high performance liquid chromatography technique (HPLC) on an Agilent 1100 with a C18 reverse column and a fluorescence detector (excitation 266 nm, emission 305 nm). Elution programs were developed using solvent A (acetonitrile and a 0.002 M KH_2PO_4 with 7% acetonitrile, pH 7) and solvent B (acetonitrile) with a flow rate of 0.5 mL min^{-1}. Quantization of glyphosate in samples was calculated by comparing the peak areas for each compound with those obtained from the injection of standard solutions after derivatization. The actual values were 97 ± 2% of their theoretical values.

2.2. Organism and culture conditions

The *C. elegans* strains used in the current study were N2 var. Bristol, CF1038 [*daf-16*(*mu86*)*I*] and TJ356 [*zIs356 IV* (*pdaf-16-daf-16*::*gfp*; *rol-6*)] obtained from the Caenorhabditis Genetics Centre at the University of Minnesota (Minneapolis, MN, USA). *C. elegans* was routinely propagated on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50 at 20 °C, using the standard method previously described by Brenner, 1974. Synchronization of worm culture was achieved by treating gravid hermaphrodites with alkaline bleach solution as described by Stiernagle (2006).

2.3. Nematode pesticide acute exposure

Age-synchronized L4-staged nematodes were treated with sublethal concentrations of glyphosate F or paraquat in M9 buffer (500 worms mL^{-1}) for 16 h at 20 °C supplemented with *E. coli* OP50 as food source (DO_{600nm} = 1). Worms exposed to M9 buffer without any herbicide were assayed as a control group and the ones exposed to paraquat as a positive control group for oxidative stress induction (Park et al., 2009). Three replicates were performed per treatment, and independent experiments were made three times. After exposure periods, the survival was always checked under a dissecting microscope by scoring the percentage of dead worms under a dissecting microscope as alive if moving or dead if unresponsive to gentle probing. None of the treatments presented a survival lower that 99%.

2.4. Reactive oxygen species detection

H2DCF-DA was used to quantify intracellular ROS in nematodes using a microplate reader (Schulz et al., 2007). Age-synchronized L4-staged N2 nematodes were treated with glyphosate F (4.8 mM) or