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Phytotoxicity of Roundup Ultra 360 SL in aquatic ecosystems: Biochemical evaluation with duckweed (*Lemna minor* L.) as a model plant

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ABSTRACT

Glyphosate-based herbicides (e.g. Roundup Ultra 360 SL) are extensively used in aquatic environment. Although glyphosate is more environmental favorable than many other herbicides, it may be exceptionally dangerous for aquatic ecosystems through high water solubility. Thus, the aim of the work was quantification of influence of Roundup Ultra 360 SL (containing isopropylamine salt of glyphosate as an active ingredient) on biomass and chlorophyll content within duckweed (*Lemna minor* L.). Moreover, changes in polyamine content and activity of such antioxidative enzymes as catalase (CAT) and ascorbate peroxidase (APX) were assayed in order to determine the biochemical mechanisms of *L. minor* response to the herbicide treatment. Obtained results showed that phytotoxicity of the herbicide was connected with decrease in chlorophyll-*a*, *b* and *a*+*b* content, and reduction of biomass growth. Roundup, similarly to some abiotic and biotic stressors, caused over-accumulation of putrescine, spermidine and total polyamines (PAs) within duckweed tissues. In addition an increase in CAT and APX activities suggested that stress generated by the herbicide treatment was at least partially connected with oxidative burst. Intensity of the duckweed responses to the herbicide was dependent on the applied herbicide level and/or duration of treatment.

1. Introduction

Roundup Ultra 360 SL is a foliar-applied, post-emergent systemic herbicide containing isopropylamine (IPA) salt of glyphosate (*N*-phosphonomethylglycine) as an active ingredient (Fig. 1). This wide spectrum herbicide is commonly used against monocotyledonous and dicotyledonous (annual and perennial) weeds on arable, non-arable lands, allotments and in forestry. The application of glyphosate inhibited the photosynthesis process within the plant tissues. It also decreased the activity of cytochrome P450, and disturbed the shikimic acid pathway as it inhibited competitively 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) [1–3]. As a consequence, the biosynthesis of aromatic amino acids and proteins was decreased. The herbicide also caused reduction of the secondary products of the shikimate pathway and an inclusion of carbon into the accumulated pool of shikimate [4]. When treated with glyphosate, the maize tissue cultures demonstrated the following features: accumulation of shikimic and quinic acids (not detected in untreated plants), decrease in ferulic/diferulic acid ratio in cell wall phenolics, as well as polyamines (PAs) and amino acids level, and increase of sugar content [5].

Although glyphosate is believed to be relatively safe for environment, since its rapid soil sorption, and thus resistance to leaching, rapid biodegradation and low toxicity to mammals, birds and fish, it may induce an abiotic stress within plants and other organisms of the ecosystem [3]. Decrease of ribulose-1,5-diphosphate carboxylase/oxygenase (Rubisko) large subunit and increase in accumulation of antioxidant enzymes, including ascorbate peroxidase (APX), glutathione *S*-transferase (GST), thioredoxin h-type, nucleoside diphosphate kinase 1 (NDPK1), peroxiredoxin and chloroplast precursor of superoxide dismutase [Cu–Zn] (SOD) within rice leaves treated with glyphosate suggests, that the herbicide generated oxidative stress within plant tissues [6]. Similar antioxidant response to the glyphosate treatment showed soybean roots and it was followed by increase in catalase (CAT) and APX activity [7]. While in leaves, the activity of guaiacol peroxidase (GOPX) was elevated and CAT activity was reduced.

Abbreviations: ALA, δ-aminolevulinic acid; AO, ascorbate oxidase; APX, ascorbate peroxidase; CAT, catalase; DAO, diamine oxidase; DHAR, dehydroascorbate reductase; EDTA, ethylenediaminetetraacetate; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GOPX, guaiacol peroxidase; GR, glutathione reductase; GST, glutathione *S*-transferase; HR, hypersensitive response; IPA, isopropylamine; MDHAR, monodehydroascorbate reductase; NDPK1, nucleoside diphosphate kinase 1; PAO, polyamine oxidase; PCD, programmed cell death; PAs, polyamines; POEA, polyoxyethylene amine; ROS, reactive oxygen species; Rubisko, ribulose-1,5-diphosphate carboxylase/oxygenase; SOD, superoxide dismutase; TMV, tobacco mosaic virus.

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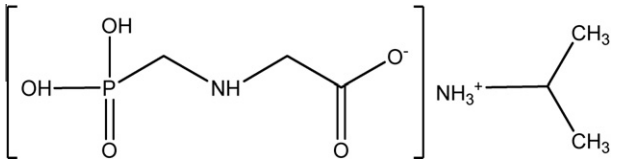

Fig. 1. Chemical structure of isopropylamine (IPA) salt of glyphosate (*N*-phosphonomethyl-glycine).

Glyphosate-based herbicides were also extensively used in the aquatic environments to destroy unnecessary flora [8]. These ecosystems are especially exposed to pollution by various pesticides, due to intentional application, aerial drift, runoff from agricultural fields and/or runoff from accidental release [9]. For example, use of copper (II) sulfate (IV) over the past century against mildew (*Plasmopara viticola*, Berk. & M.A. Curtis) and other fungal diseases on vineyards caused an increase of copper (II) pollution into aquatic environments [10]. According to Mateos-Naranjo et al. [11], although glyphosate is more environmental favorable than many other herbicides, it may be dangerous for aquatic ecosystems through high water solubility (68.6 mM = 11.6 g × dm^{-3}). Tsui and Chou [8] showed that glyphosate toxicity towards various water organisms depends on its formula and applied doses in different commercial products. Against bacterium *Vibrio fischeri* (Beijerinck), microalgae *Selenastrum capricornutum* (Printz) and *Skeletonema costatum* (Greville), protozoa *Tetrahymena pyriformis* (Ehr.) and *Euplotes vannus* (Muller), and crustaceans *Cariodaphnia dubia* (Richard) and *Acartia tonsa* (Dana) Roundup and its surfactant polyoxyethylene amine (POEA) were more toxic than IPA salt of glyphosate and glyphosate acid. Microalgae and crustaceans were 4–5 folds more sensitive to the herbicides than bacterium and protozoa, and Roundup toxicity to *C. dubia* was elevated by increase of pH value and suspended sediment concentration. On the other hand, glyphosate harmfulness to freshwater green alga *Scenedesmus quadricauda* (Turbin) was connected with reduction of its growth, photosynthesis and chlorophyll-*a* synthesis by 2 mg × dm^{-3} of herbicide, and complete inhibition of these processes by the herbicide concentration equal to or higher than 20 mg × dm^{-3} [2].

Duckweed is a model plant, often used in ecotoxicology for evaluation of pesticide phytotoxicity. Its usefulness is due to its small size, rapid growth, easiness to cultivate and sensitivity to a wide range of xenobiotics [12]. The influence of some xenobiotics on such parameters as morphological characteristics, growth, photosynthesis, biomolecules synthesis was examined by means of duckweed bioassays [13]. For example, it was demonstrated that copper (II) and flumioxazin (phenylpyrrole herbicide), applied respectively in 200 and 1 μg × dm^{-3} doses, decreased photosynthesis yield in duckweed as early as 24 h [10], while fungicides copper and folpet, induced CAT activity [14]. However, there are only a few data about *L. minor* response to glyphosate-based herbicides. Thus the aim of the work was to quantify the Roundup Ultra 360 SL effect on biomass and chlorophyll content within duckweed tissues. In addition, its effect on accumulation of polyamines, and CAT and APX activity after the herbicide treatment was determined.

2. Material and methods

2.1. Plants

The duckweed was cultivated in a climatic chamber at 22–24 °C and the photoperiod being 16L:8D. Plants were grown in modified Hoagland's medium prepared in accordance to Nielsson [15], with an addition of Roundup Ultra 360 SL (Monsanto, Poland) at concentration of 1.58, 3.16 and 31.58 mmol IPA salt of glyphosate × dm^{-3} of medium or without herbicide (control). The plant material was collected after 4, 8, 24 and 48 h for enzymatic analysis; after 24, 48 and 96 h for polyamine assays and after 3 weeks for measurement of biomass and chlorophyll content. The assays were performed with use of fresh plant material, while plant used for polyamine assays were early lyophilized.

2.2. Estimation of duckweed growth

Freshly collected plant material was dried on filter paper and weighed on the balance WPA 120/C/1 type (Radwag, Radom, Poland). The influence of Roundup on growth of duckweed was determined by comparison of the biomass of control plants and herbicide-treated ones.

2.3. Chlorophyll assay

Content of chlorophyll-*a*, *b* and sum of *a*+*b* was assayed according to method described by Lichtenthaler [16]. Fresh plant material (0.5 g) was homogenized with 25 cm^3 of 80% acetone. Absorbance of the obtained supernatant was measured with Spectrophotometer Specol 11 type (Carl Zeiss, Jena, Germany) at 645 and 663 nm. Chlorophyll content was calculated using following equations:

$$C_a = 12.7 \times A_{663} - 2.69 \times A_{645}$$
$$C_a = 22.9 \times A_{645} - 4.68 \times A_{663}$$
$$C_{a+b} = 20.2 \times A_{645} - 8.02 \times A_{663}$$

where c_a, c_b and c_{a+b} is content of chlorophyll-*a*, *b* and *a*+*b* respectively, A_{663}, A_{645} – absorbance at 663 and 645 nm.

2.4. Enzyme assays

CAT activity was determined with Chance and Maehly [17] method. Fifty milligram of fresh plant material was homogenized with 0.05 M phosphate buffer pH 7.0 and obtained suspension was centrifuged at 15,000 *g* for 20 min at 5 °C. The supernatant (0.05 cm^3) was dissolved with 2.95 cm^3 of 0.015 M H_2O_2 in phosphate buffer pH 7.0. An absorbance at 240 nm was measured with Spectrophotometer Cecil CE 102 (Cecil Instruments, Cambridge, England) at the beginning of enzymatic reaction and after 3 min. Unit of CAT activity was calculated with assumption that absorbance decrease $\Delta A = 0.0055 \times min^{-1}$ is equal 1.25 unit.

APX activity was assayed according to method developed by Nakano and Asada [18]. Fresh plant tissue (0.2 g) was homogenized in 5 cm^3 of 0.05 M phosphate buffer pH 7.6 with addition of ethylenediaminetetraacetate (EDTA) (29 mg × 100 cm^{-3}), sorbitol (20 g × 100 cm^{-3}), polyvinylpyrrolidone (2 g × 100 cm^{-3}) and ascorbate (17 mg × 100 cm^{-3}) and centrifuged at 15,000 *g* for 20 min at 5 °C. 0.05 cm^3 of the supernatant or distillated water (control) was mixed with 1.95 cm^3 of mixture, containing 100 cm^3 of 0.05 M phosphate buffer pH 7.0, 10 mm^3 of 0.1 mM H_2O_2 and 252 mg of pyrogallol. An increase in absorbance at 430 nm, after 1, 2 and 3 min of enzymatic reaction was determined using spectrophotometer Cecil. Unit of APX activity was calculated using the following equation:

$$U = \frac{\Delta A - \Delta A_0}{2.47} \times 20$$

where ΔA is increase in absorbance of sample containing extract of enzyme, ΔA_0 is increase of absorbance in control, 20 is dilution coefficient, 2.47 is milimolar absorbance coefficient.

CAT and APX activity were expressed in units (U) per 1 mg of protein. Protein quantity within the enzymatic extracts was determined according to Bradford [19].