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Original Research

Application of White Mustard (*Sinapis alba*) Biotest in the Assessment of Environmental Contamination by Glyphosate

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Abstract

The aim of our experiment was to examine the influence of glyphosate on photosynthesis in plants and to check the usefulness of *Sinapis alba* for monitoring water contamination by glyphosate. The experiment was carried out in a phytotron chamber. It was divided into two parts. The first one determined the effect of a glyphosate herbicide (Glyfos 360 SL) on chlorophyll "a" activity in white mustard (*Sinapis alba*) seedlings placed in water. The second part assessed the white mustard response to contamination (by glyphosate) of water originating from the first part of the study. Changes in chlorophyll "a" activity occurred within 24h exposure of the plants to the herbicide. In the second part of the research, inhibition of over 47% of white mustard root growth was found as a result of 3-day exposure to water in which the test plant seedlings had been placed, following 48h-hour treatment with glyphosate herbicide at 7.2 ml/l concentration. This indicates the high utility of white mustard (*Sinapis alba*) in the assessment of environmental contamination by glyphosate.

Keywords: white mustard, chlorophyll "a," glyphosate, water contamination, Phytotestkit

Introduction

One of the consequences of chemical protection of plants is contamination of the environment by pesticides [1]. In spite of registering compounds with more favourable toxicological parameters, those substances still provide a threat for organisms in the biocoenoses [2]. This particularly concerns glyphosate (N-[phosphonomethyl]glycine). This herbicide, used since 1974, plays a dominant role in the global market of crop protection chemicals [3]. Glyphosate is taken up by the above-ground parts of plants and migrates down through the xylem and floem to roots [4]. Its mode of action consists in blocking the activity of shikimic acid (EPSP synthase), which is necessary in the process of biosynthesis of amino acids: phenylalanine, tryptophan, and tyrosine [5]. This leads to inhibiting synthesis of proteins and many secondary metabolites, such as growth stimulators and inhibitors, lignins, flavonoids, anthocyanins [6, 7]. The latest research suggests that glyphosate also influences the process of photosynthesis [8, 9].

It has been demonstrated that glyphosate as a stress factor causes changes in the permeability of cell membranes and disturbs electron transport in photosystems I and II. This results in changes in chlorophyll fluorescence, considered to be a specific biomarker of plant stress [10]. On the other hand, stimulation of the process of photosynthesis was observed under the influence of sublethal, reduced doses of glyphosate [11]. As results from the research conducted, glyphosate can be subject to decomposition in plants to nitrates and carbon monoxide [12], but the main protective mechanism of plants is to expel it by the roots [13, 14]. As a consequence of these processes, the growth

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of accompanying plants can be inhibited [15]. However, herbicide uptake through the roots is low, since the active substance is subject to strong soil sorption, which reduces its availability to plants [16]. The half-life period of the compound under such conditions is 8-30 days [17]. The rate of glyphosate decomposition depends on its microbiological activity [18, 19]. The main metabolite of the herbicide, aminomethylphosphonic acid (AMPA), does not demonstrate phytotoxic properties [20, 21].

Glyphosate is also subject to rapid decomposition in a water environment [22, 23], and its half-life in water is about 4 days to 3-4 weeks [6]. According to previously conducted research, glyphosate shows low toxicity to animals [24, 25]. However, more recent experiments demonstrate the need to verify those views. This can be exemplified by threats posed by herbicides containing this active substance to frogs [26] and fish [27]. An assessment of environmental contamination by glyphosate is therefore of high importance for monitoring exotoxic threats. With this aim in view, biological methods are increasingly applied. They are based on the assessment of the response of test organisms to chemical stresses. Among others, changes in chlorophyll fluorescence and inhibition of plant germination and growth can be used as chemical stress indicators.

Research into this issue was carried out with the use of seeds (Phytotestkit test procedure) and young seedlings of white mustard (*Sinapis alba*) to examine the influence of glyphosate on photosynthesis in plants and to check usefulness of *Sinapis alba* for monitoring water contamination by glyphosate.

Material and Methods

Preliminary Research

The experiments were carried out in six replications in a phytotron chamber under controlled temperature (24ºC – 12h + 16ºC – 12h), light (24,000 lux) and air humidity (60%) conditions. The research was carried out pursuant to the Phytotestkit test procedure (distributor: TIGRET sp. z o o., Warsaw, manufacturer: MICROBIOTESTS Inc., Belgium). White mustard (*Sinapis alba*) seeds were exposed to glyphosate (Glyfos 360 SL, manufactured by Cheminova Polska Sp. z o.o., with 36% glyphosate content) in the concentration range of 3 µl/l to 3.6 ml/l (Fig. 1), after which the concentration of the herbicide resulting in 50% inhibition of white mustard root growth was determined (IC_{50}).

Part I
The Assessment of the Effect of Glyfos 360 SL Herbicide on Chlorophyll "a" Fluorescence in White Mustard Seedlings

The experiment was carried out in six replications in a phytotron chamber, with identical parameters of temperature, light and humidity as in the preliminary research. The research material were seeds of white mustard, *Sinapis alba*, subject to germination on wet filter paper. Young 6-day seedlings of the test plant were then placed in 100 ml beakers, 5 plants in a beaker, on cotton gauze, providing the roots with access to water (Fig. 2). In order to ensure proper conditions for plant growth, a liquid universal fertilizer was added in a dose of 5 ml/1 litre of water (NPK: 6%-4%-6%). After 7 days, water was replaced to eliminate the effect of mineral fertilization on the effect of the herbicide. The plants prepared in this way were treated with a water solution of Glyfos 360 SL herbicide. The preparation, dissolved in water, was applied on cotyledons with the use of a micropipette, in the amount of 50 µl of the liquid per plant. The herbicide product was applied at the following concentrations: 0.5% (1.8 ml glyphosate in 1 litre of water), 1.0% (3.6 ml/l) and 2.0% (7.2 ml/l). Two, 24 and 48h after application of the herbicide, chlorophyll "a" fluorescence was measured using a Handy PEA meter, Hansatech Instrument, UK. The Fv/Fm parameter (maximum quantum efficiency of photosystem II) was used to assess chlorophyll "a" activity.

Part II
Assessment of the Influence of Water Contaminated with Residues of Glyfos 360 SL Herbicide on Germination and Development of White Mustard Roots

The experiment was carried out in six replications, with the application of a standard Phytotestkit test, using the water contaminated with Glyfos 360 SL herbicide-water (with no herbicide) in which plants (with herbicide applied on cotyledons) were growing was used in this part. Three and 7 days after seed incubation, the effects of water contamination on germination and the length of test plant roots were measured (with the use of the Image Tool software).

All results of the research were compiled and statistically compared using the Duncan test at the significance level of 0.01.

Results

As results from the data presented (Fig. 1), an IC_{50} dose inhibiting the growth of the test plant roots, after 3 days of plant exposure to Glyfos 360 SL, was 9.5 µl/l glyphosate

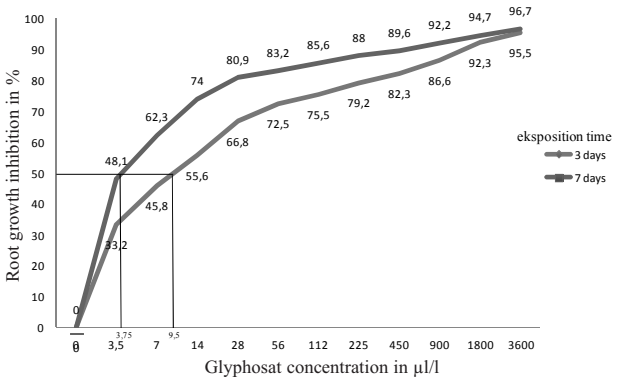

Fig. 1. The effect of the difference glyphosate concentration (Glyfos 360 SL) on root growth inhibition of white mustard (*Sinapis alba*) – determination IC_{50} parameter.

Table 1. The effect of herbicide on Fv/Fm parameter value in white mustard (*Sinapis alba*) cotyledons.

Concentration of Glyfos 360 SL herbicide	Exposure time of tested plant cotyledons on herbicide		
	2 hours	24 hours	48 hours
Controls	0.827 a	0.823 a	0.810 a
0.5%	0.810 a	0.786 b	0.752 b
1.0%	0.789 a	0.756 b	0.707 b
2.0%	0.795 a	0.692 c	0.631 c
$LSD_{0.01}$	0.039	0.033	0.050

Means in the same column with common letter are not significantly different (P<0.01).
LSD – least significant differences

and 3.75 µl/l after 7-day exposure. The first significant changes in chlorophyll "a" fluorescence were observed after 24h exposure of plants to all applied concentrations of Glyfos 360 SL herbicide (Table 1). The values of this factor decreased with the growth of the herbicide concentration and lengthening the exposure period from 24 to 48h. The highest drop in chlorophyll "a" fluorescence was observed at a herbicide product concentration of 7.2 ml glyphosate in 1 liter of water. Two parameters were examined as the plant response to water contamination: germination of seeds and inhibition of the growth of white mustard (*Sinapis alba*) roots.

The results obtained for germination of the test plant seeds indicate a lack of the effect of water contamination by residues of Glyfos 360 SL herbicide, regardless of the period of water exposure or the assessment time (Table 2). Another parameter of the assessment, the length of white mustard roots, was determined 3 and 7 days after establishing the Phytoteskit test (Table 3). The response of the plants to water contamination was observed for all examined objects, i.e. with the previous application of the herbicide at 1.8 ml/l, 3.6 ml/l, and 7.2 ml/l. The first significant changes in the length of roots occurred three days after treating the seeds with water from the 48h exposure to the residues of the herbicide expelled by plant roots. Reduction of the root length in water contaminated by residues of glyphosate herbicide applied in the first experiment at a concentration of 7.2 ml/l amounted in this case to 45.7%. Therefore, it was slightly below the IC_{50} value determined in the preliminary research (Fig. 1). With shorter exposure periods, i.e. after 24 and 2h, phytotoxic effects occurred after a longer, 7-day period of treating the seedlings with contaminated water (Table 3). This means that the degree of inhibiting roots of the test plant seedlings was related to the dose of herbicide expelled by roots of the test plant in experiment 1, and later taken up by subsequent plants in experiment 2.

Discussion

The effect of glyphosate on the process of photosynthesis, demonstrated in this research, is confirmed in the cited literature [8, 28, 29]. The experiment conducted demonstrated that changes in chlorophyll "a" fluorescence are directly proportional to the applied concentration of Glyfos 360 SL herbicide concentration (Table 1). A drop in chlorophyll "a" photosynthetic activity as a result of 24h exposure of the white mustard cotyledons to the glyphosate herbicide was already observed at the herbicide concentration of 1.8 ml/l (according to information on the product label it is equal to a 2l/ha dose in 400 l of water). An increase in the herbicide concentration to 3.6 ml/l and 7.2 ml/l resulted in further reduction of the value of the examined parameter. Similar effects of glyphosate resulting in deterioration of photosynthesis parameters were observed in experiments concerning transgenic soya. This resulted in diminishing the leaf-area and reducing the biomass of plants [9]. Olesen et al. [10] observed changes caused by glyphosate in the process of photosynthesis on the basis of two parameters – gas exchange and chlorophyll fluorescence. Higher differences were observed while examining the effect of various glyphosate concentrations on gas exchange. With the increase of herbicide concentration, a significant decrease in binding carbon dioxide by plants occurred, with simulta-

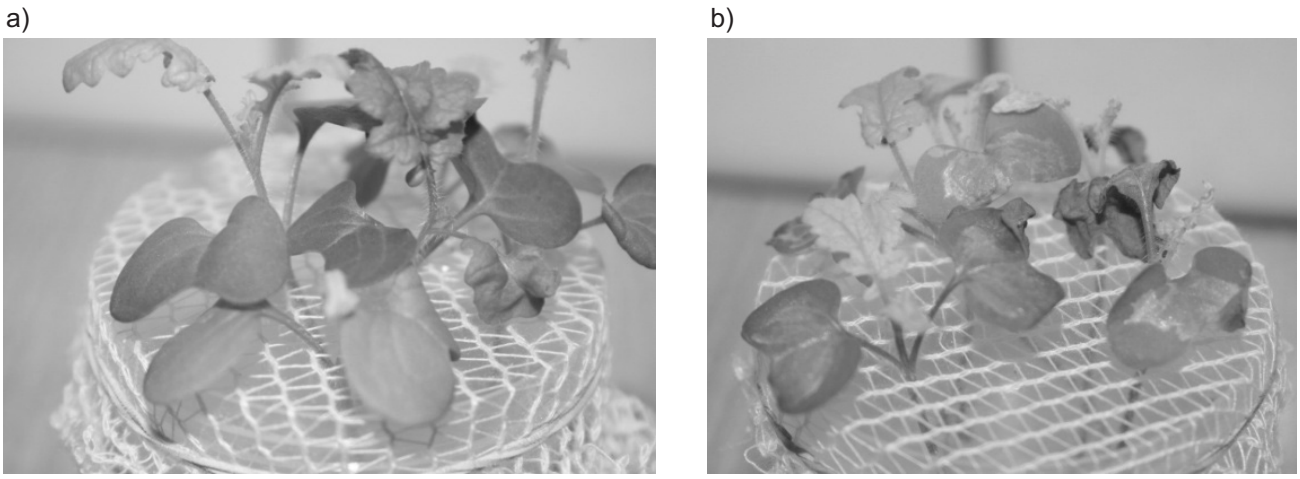

Fig. 2. Seedlings of white mustard placed in beaker. a) control, b) seedlings after 48 hours treatment with 2% Glyfos 360 SL herbicide (7.2 ml glyphosate/l of water).

Table 2. The effect of water contamination by residues of Glyfos 360 SL herbicide on germination of white mustard (*Sinapis alba*) seeds (%).

Concentration of Glyfos 360 SL herbicide applied on seedlings of tested plant	Period of water exposure to plant roots treated by Glyphos 360 SL herbicide					
	2 hours		24 hours		48 hours	
	Percent of germinated seeds					
	after 3 days	after 7 days	after 3 days	after 7 days	after 3 days	after 7 days
Controls	95.0 a	100 a	100 a	100 a	98.3 a	98.3 a
0.5%	95.0 a	96.7 a	96.7 a	98.3 a	96.7 a	98.3 a
1.0%	98.0 a	98.0 a	96.0 a	98.0 a	100 a	100 a
2.0%	96.7 a	98.3 a	96.7 a	98.3 a	98.3 a	98.3 a
$LSD_{0.01}$	12.17	6.31	8.92	5.76	6.31	5.76

Means in the same column with common letter are not significantly different (P<0.01).
LSD – least significant differences

Table 3. The effect of contamination of water by residues of Glyfos 360 SL herbicide on length of white mustard (*Sinapis alba*) roots (%).

Concentration of Glyfos 360 SL herbicide applied on seedlings of tested plant	Period of water exposure to plant roots treated by Glyphos 360 SL herbicide											
	2 hours				24 hours				48 hours			
	Root length											
	after 3 days		after 7 days		after 3 days		after 7 days		after 3 days		after 7 days	
	mm	%	mm	%	mm	%	mm	%	mm	%	mm	%
Controls	30.4 a	100	77.6 a	100	28.5 a	100	74.9 a	100	28.0 a	100	79.8 a	100
0.5%	27.6 a	90.8	72.1 a	92.9	24.4 a	85.6	64.8 a	86.5	24.9 ab	88.9	65.9 b	82.6
1.0%	26.9 a	88.5	69.9 a	90.1	28.1 a	98.6	70.2 a	93.7	23.3 ab	83.2	58.5 b	73.3
2.0%	23.4 a	77.0	51.7 b	66.6	20.9 a	73.3	50.9 b	68.0	15.2 b	54.3	35.2 c	44.2
$LSD_{0.01}$	9.72	31.9	15.28	19.7	7.93	27.8	11.76	15.7	9.72	34.7	12.29	15.4

Means in the same column with common letter are not significantly different (P<0.01).
LSD – least significant differences

neous lower changes in chlorophyll fluorescence. Other research reported that even the application of glyphosate on green parts of stems can result in decreasing the chlorophyll content in young leaves that were directly exposed the substance [8]. It was also found that the application of glyphosate in a dose of 0.28 kg/ha caused reduction of the chlorophyll content and a decrease in the dry matter content in soya [29]. This means that (as observed in the current study) shortly after the treatment, a decrease in chlorophyll "a" fluorescence can provide a good indicator of plant contamination by glyphosate. It was also demonstrated in this study that water contamination by this herbicide did not result in inhibition of germination of white mustard seeds. No influence of glyphosate on seed germination was proven by Morash R. and Freedman B. [30], or Piotrowicz-Cieślak et al. [31]. However, it was found that this herbicide could have a negative effect on seed germination as a result of treatment performed during the plant maturing stage [32, 33]. Inhibition of the growth of white mustard roots in experiment 2 provides direct evidence of water contamination by residues of glyphosate expelled by test plants, and was related to the concentration of the herbicide applied.

It is well-known that absorption and movement of herbicide increases with the growth of concentration of the applied agent [34]. The effects observed indicate the threat of phytotoxicity, not only as a result of glyphosate uptake by plant leaves (which is commonly known) but also through their roots. The research carried out by Piotrowicz-Cieślak et al. [31] proved that plants can demonstrate sensitivity to contamination of soil with glyphosate by root growth inhibition and by production of a stress factor – myo-Inositol. This confirmed an increased inhibition of root lengthening along with an increase of glyphosate concentration in soil. In other studies [35], a close correlation was found between the response of young maize seedlings and the quality of glyphosate absorbed from the ground. A stimulating effect was observed with smaller amounts of the substance absorbed, while inhibition of plant growth occurred for larger amounts. Thus, bio-accessibility of this substance through the root system can pose a threat to the

proper development of successive plants. This is proven by research carried out by Bott S. et al. [36], who demonstrated inhibition of soya development after soil application of glyphosate at a dose of 720-1440 g/ha.

Conclusions

1. The first symptoms of glyphosate phytotoxic effects in the form of reduced chlorophyll "a" fluorescence in white mustard cotyledons were observed after 24h as of herbicide application.
2. Glyphosate doses expelled after the treatment through plant roots can cause inhibition of the growth of white mustard (*Sinapis alba*) seedlings used as a test plant.
3. The research (including the determination of the IC_{50} value) demonstrated the high utility of white mustard (*Sinapis alba*) for assessing environmental contamination by glyphosate.

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