The glyphosate influence on cytogenetic and biochemical aspects of wheat (Triticum aestivum L) seedlings development

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Abstract. The influence of glyphosate on the germination and development of bread wheat (Triticum aestivum) was researched. The chronical influence during all the time of growth was estimated, together with the consequences of the contact with glyphosate of the already formed seedlings. A dose-dependent decrease in the growth rate of seedlings in the presence of glyphosate was found, and even in normally developing seedlings damage to the root tips was observed. In particular, in seedlings, the integrity of the root cap was disrupted, degenerative changes of the rhizodermis and root hairs were detected. It has been shown that a decrease in the root growth rate during chronic exposure is largely due to the suppression of mitoses. In addition, high glyphosate concentrations had a general toxic effect, stopping the development of seedlings yet at the stage of primary roots growth through enlarging the cells. It was found that the antioxidant system of seedlings prevented the development of oxidative stress at the concentration of herbicide 50 μM, in particular, due to the activation of peroxidase. Placing seedlings grown on distilled water in 100 μM glyphosate solution induced lipid peroxidation. Chronic effects of herbicide in this concentration inhibited lipid metabolism in general.

1. Introduction
Systemic herbicide glyphosate, N-phosphonomethyl derivative of the amino acid glycine (N-(phosphonomethyl)-glycine) is an active element of the medicines “Roundup”, “Ranger”, “Alligare”, “Glynar”, “Reminder” and so on. The wide-range use of glyphosate-based herbicides is presupposed by the selection of resistant transgene varieties of soy, colza, maize, cotton, sugar beet [1], that enables us to root out weeds damage-free to agricultural species. Also glyphosate is used as a desiccant in 10 – 15 days before harvesting in order to dewater the plant tissue, that enhances their maturation and decreases the general humidity of the corn [2].

Penetrating through the leaves, glyphosate spreads through the plant and concentrates in meristematic tissues [3]. The effect shows itself after several days: the leaves wrinkle, chlorosis appears, photosynthesis decreases, necrosis of meristems is expressed [4]. The action of glyphosate is
based on inhibition of enzyme 5-enolpiruvil-shikimate-3-phosphate-synthase of the shikimate pathway in the biosynthesis of benzoid aroma compounds. As a result, the synthesis of chorismate is broken, while the former is the precursor of a whole number of compounds, including aromatic amino acids: phenylalanine, tyrosine and tryptophan. At the same time, in some plants the effect of herbicide develops without clear deficit of aroma amino acids [5]. Officially glyphosate is not referred to as a carcinogen and a mutagen [6], although there are data about the ability of this herbicide to cause genotoxicity [7].

Glyphosate penetrates the plants through the root system from the soil, where it enters both immediately while processing, and with the run-off waters from the neighboring fields. To additional sources belong root excreta of glyphosate-resistant plants, as well as the plants, died in processing. In spite of rather quick destruction of the present compound, glyphosate can remain to a certain extent in the soil by the time of planting after the pre-planting processing of the fields, especially while breaking the recommended terms of processing (2 – 4 weeks before planting). It should be noted here that the period of glyphosate half-life depends on climatic conditions and the type of the soil, and varies from 3 to 141 days [8], while the products of decay preserve in the soil up to 2 – 3 years [9]. Soil particles adsorb herbicide to a significant degree, meanwhile glyphosate presence in the soil causes the decrease in the plant growth rate, leads to withering, chlorosis, higher penetrability of cell membranes in root tissues, disruption in the development of side roots. Besides, while administering phosphoric fertilizers, the unrelated glyphosate concentration in soil increases due to phosphorus and glyphosate rivalry for adsorption place [4].

The aim of the present paper was to study the influence of glyphosate on the bread wheat seedlings (Triticum aestivum L.) in penetrating through the root system.

2. Materials and methods
In the work we used the seeds of bread spring wheat of the variety “Omsk 35”. The planting material was anticepticized by the 96 % ethanol for 3 minutes, then it was thrice exposed to the sterilized distilled water.

The seeds were grown in the distilled water in glyphosate solutions 100 and 50 μM thick at 25°C. The germination was determined on the 5th day. The normally germinated seedlings were considered those with, at least, two roots twice the size of the seed and the stalk more than a half of the seed length, with the leaf seen under the coleoptile.

Peroxidase activity in plants was identified by the rate of orthophenylnediamine oxidation. The extract for activity analysis was received through homogenizing of 20 mg of seedlings roots in 4 ml of phosphate buffer (pH 5.8). Then the homogenate was filtered, then – centrifuged for 5 min at 12000 g. The analyzed reaction mix contained 0.1 ml of the gotten extract, 0.1 ml 6 μM H₂O₂ and 0.1 ml of orthophenylnediamine solution (6 μM). The reaction was stopped after 30 secs by the addition of 0.1 ml 2M H₂SO₄. Optical density of the solution at 458 nm was measured at spectrophotometer (UNICO 2800, USA).

In order to determine the ratio of diene conjugates in the sample we carried out the extraction with the mixture of hexane with isopropanol [10]. For this purpose, 30 mg of roots were homogenized in 1 ml of the named mixture (for 1 min.) and extracted by vortexing for 10 min. The received mixture was centrifuged for 5 min at 12000 g. For the division of hexane and isopropanol phases we added 0.5 ml of distilled water into supernatant and again centrifuged it under the same conditions. The upper heptane phase was dissolved 5 times by the 95% of ethanol and was analyzed with the help of spectrophotometer (UNICO 2800) against field-blank sample. Taking into consideration that optical density (D) at the wave length 215 nm reflects the contents of isolated double relations, and with 232 nm – the contents of diene conjugates, we calculated the number of oxidation products in unsaturated fat acids in terms of oxidative index, as a ratio D$_{232}$/D$_{215}$ [11].

In determine the mitotic index, the roots were fixed for a day in the mixture of glacial acetic acid with 96% ethanol (1:3). Then they were placed for 10 min into 1M solution of HCl at 60°C, the root tip was cut off, dyed for 10 min by acetoarmine at 90°C and was squashed beneath a cover glass. The
mitotic index was calculated as the percentage of cells in different stages of mitosis to the general number of meristematic cells [12].

The microscopic research was carried out with the microscope Primo Star (Carl Zeiss MicroImaging, Germany), microphotographs were made with the digital camera Axiocam 105 color.

3. Results and discussion

Glyphosate in the solution of 50 μM thick practically did not inhibit the process of germination in bread wheat seeds, but afterwards it stopped the growth of some seedlings (figure 1).

![Figure 1](image1.png)

**Figure 1.** Assessment of the seeds T. aestivum germination 1 – normal seedlings, 2 – the growth stopped on the stage of the seedling development, 3 – no germination.

The solution of 100 μM thick caused damage in the simultaneous germination – as a rule, the seedlings catching up stopped in their development on the second day. On the third day of the crop cultivation grown in the media, containing glyphosate in the studied proportion, they lagged behind significantly, comparing with the test group. The further growth of the roots and the coleoptile correlated with the proportion of glyphosate in the medium (figure 2).

![Figure 2](image2.png)

**Figure 2.** The influence of glyphosate on the seedlings T. aestivum development. a – root, b – coleoptile. 1 – distilled water, 2 - 50μM, 3 – 100μM.

The microphotos of the roots in 3-day seedlings showed that already at the concentration of 50 μM there is the damage of root tips, especially noticed were the damages to the rhizodermis and the root
cap (figure 3). These changes are probably the result of non-special genotoxicity, because the damage to the root tip is noticed also with the herbicide influence of the sulfonylurea herbicides [13]. At the same time, it is possible that the specificity in damaging the roots with glyphosate is one of the causes of increased susceptibility in plants to pathogens, while processing with the present herbicide [14].

Figure 3. The root tip of the seedling T. aestivum. a – distilled water, b – 50 μM thick solution of glyphosate.

Because the study of the short-term exposure to glyphosate in the buckwheat seedlings roots (Fagopyrum esculentum Moench) and the beans (Vicia faba L.), we found out the dose-dependent decrease in mitotic index already after 3 hours [15], [16]. The mitotic activity of meristematic cells in bread wheat seedling roots was identified in growing plants under the influence of glyphosate. The research of mitotic activity of meristematic cells in the seedling roots on the third day showed that the used proportions of glyphosate suppressed cell division. With glyphosate concentration in the medium of 50 μM, the mitotic index on the third day of growth decreased by 6 times compared to that of the control, at a concentration of 100 μM - by 10 times.

The decrease in mitosis frequency under the influence of glyphosate was noticed not only with the plants, but also on the eggs and embryos of the sea urchin (Sphaerechinus granularis Lamarck), where herbicide lead to the decrease in the level of DNA synthesis in the S-phase of the cell cycle, with the following stop as a result of incomplete replication in G2-phase [17]. These data enable us to suppose that the effect of mitosis suppression is connected not only to the blocking of the shikimate way, because animals do not have it.

It is known that the first mitosis in wheat is detected at the root length of about 10 mm that corresponds to the 2 – 3 day seedlings, while before this time the root grows by enlarging the cells [12]. Comparing the data about the changes in the growth rate under herbicide influence with the decrease in mitotic index, it is possible to suppose that at the concentration of 50 μM the decrease in the rate of the root growth on the third day is largely determined by mitosis suppression. At the concentration of 100 μM, the lagging behind in the growth of some plants took place already at the stage of germination during the second day. There are facts that at the high ratio of glyphosate the decrease of mitotic index is related to the necrosis of meristematic cells [16]. In our research there was not found the death of meristematic cells. It can be supposed that the lagging behind in the growth under these conditions is related mostly not to the suppression of cell division, but to the general genotoxicity, leading to the suspense in development.

As a rule, stress influences lead to the break of balance between the rate of active forming of reactive oxygen species in the cells (ROS) and the rate of their inactivation by the enzymes low molecular weight compounds. The oxidative stress, forming in these conditions, expresses itself in the development of chain reaction of lipid peroxidation, protein and DNA damage. It is supposed that glyphosate leads to the development of oxidative stress as a secondary effect, caused by the breaking of cell metabolism as a result of the shikimate way blocking in the biosynthesis benzoid aroma compounds [18].
Cell resistance to the oxidative stress is determined by the possibility of activation and the reserves of antioxidant cell systems. Thus, according to the literature sources, glyphosate processing of some plants leads to the increase in the concentration of low-molecular antioxidants (proline, glutathione, thioredoxin of h-type) and the growth activity of antioxidant system enzymes (catalase, ascorbate peroxidase, guaiacol-peroxidase, Glutathione-S-transferase, glutathione-reductase, peroxiredoxin, etc.) [19], [20], [21]. The information about the cell increase of H$_2$O$_2$ and lipid peroxidation under the influence of glyphosate are ambiguous in the literature. Thus, rice plants (Oryza sativa L.) have an increase in the proportion of H$_2$O$_2$ and malonic dialdehyde, which is the product of lipid peroxidation when leaf-treating leaves with a herbicide [19]. While influencing the potatoes seedling roots (Solanum tuberosum L.) an increase of H$_2$O$_2$ and of malonic dialdehyde in leaves, without the change in concentration in the roots [3]. At the same time, soy plants (Glycine max L.) exposure to glyphosate did not lead to the change of lipid peroxidation level [22].

In our research we studied the activity of peroxidase and the change in the content of lipid peroxidation products under the influence of glyphosate in wheat seedling roots. Diene conjugates, the primary product of lipid peroxidation, are formed while regrouping double relations in polyunsaturated fat acids under the influence of the oxygen radical. The maximum of diene conjugates absorption ranges 231-235 nm [11].

He peroxidase activity in 3-day seedlings, grown under the influence of glyphosate (50 μM), was by 1.8 times higher than in the test group seedlings that probably prevented the damage to the lipids, because the oxidative index corresponded to that in the control. At a herbicide concentration of 100 μM there was noticed the decrease in absorption by 25% at both 215 nm and 232 nm, without the change in oxidative index. On the other hand, if 3-day seedlings, grown in distilled water, were placed into the solution of glyphosate, then in a day the oxidative index at the concentration of 100 μM rose by 2 times. At a concentration of 50 μM, the oxidation index did not change. Peroxidase activity in each of the concentrations per day of influence increased by 30%. Thus, the oxidative damage to the lipids was detected only while processing the roots of 3-day seedlings, not contacting with herbicide before. The plants developing under glyphosate influence, by the third day had their oxidative index corresponding to the control values. Probably, under the chronic influence the activation of antioxidative system can significantly prevent the damage to the lipids.

So, glyphosate in the medium of growing decreases the germination of seeds and the rate of seedlings development, leads to the damage of roots and decrease in mitotic index. The development of oxidative stress is detected only at the initial stage of herbicide influence, later the activation of the antioxidant system stabilizes the situation.

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