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LONG-TERM EFFECT OF N-(PHOSPHONOMETHYL)GLYCINE ON THE DEVELOPMENT OF RHODOTORULA SPP.

A.V. TYKHENKA, L.M. VASINA

Yuriy Fedkovych Chernivtsi National University
Kotsyubynsky 2, Chernivtsi, 58012,
e-mail: l.vasina@chnu.edu.ua

The control of invasive plant species in agroecosystems is mostly associated with the use of synthetic herbicides due to their high effectiveness. One of the most commonly used is glyphosate [N-(phosphonomethyl)glycine]. It is a non-selective systemic herbicide with a broad spectrum of activity used to combat annual and perennial plants. This compound acts by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which plays a key role in the shikimate pathway and is responsible for the synthesis of an intermediate product in the biosynthesis of aromatic amino acids. N-(phosphonomethyl)glycine demonstrates an ambiguous effect on soil microbial communities. Some concentrations of the herbicide can stimulate microbial activity, increasing the diversity and complexity of the network. In other cases, the herbicide has a negative effect on the survival and structure of soil microorganisms. The effect of the herbicide on microscopic fungi remains little known, in particular on representatives of the genus Rhodotorula, which are characterized by a wide distribution in various ecological niches, high metabolic activity (synthesis of pigments, enzymes, exopolysaccharides, ergosterol), the ability to exist in microbial consortia, and adsorption capacity.

The study investigated the effect of the herbicide N-(phosphonomethyl)glycine on the physiological and biochemical parameters of two yeast species: R. rubra and R. minuta. During the study, changes in growth characteristics under prolonged herbicide exposure, carotenoid content, and the level of thiobarbituric acid-reactive substances were determined.

It was found that N-(phosphonomethyl)glycine at the highest of the tested concentrations had a dose- and time-dependent inhibitory effect on the development of carotenoid-producing yeasts, inducing oxidative stress, which was accompanied by the accumulation of lipid peroxidation products and changes in the level of major carotenoids. At the same time, a decrease in the culture density of both studied species and a reduction in the number of planktonic colony-forming units were observed. R. minuta exhibited greater sensitivity to the pollutant, as confirmed by more drastic changes in the studied parameters – a significant reduction in the number of viable cells, a multiple increase in the level of end products of lipid peroxidation, and a decrease in carotenoid levels.

Keywords: N-(phosphonomethyl)glycine, Rhodotorula spp., fungitoxicity, dose dependence

Introduction. The widespread and prolonged use of herbicides leads to their ubiquitous accumulation in the environment. In recent years, serious concerns have arisen regarding the harmful side effects of N-phosphonomethylglycine and AMPA (aminomethylphosphonic acid), which is its primary metabolite, on the life processes of plants, animals, microorganisms, and humans, since this systemic herbicide has a broad spectrum of activity and is used to combat annual and perennial weeds. The biocidal activity of N-(phosphonomethyl)glycine is associated with the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which blocks the sixth reaction in the shikimate pathway and is essential for the synthesis of aromatic amino acids and secondary metabolites that perform protective functions in plants and many microorganisms (Ruuskanen et al, 2022; Van Bruggen et al, 2018).

Despite the considerable interest of the scientific community in the problem of environmental contamination by herbicides, the mechanisms of their interaction with microorganisms, particularly yeasts, remain insufficiently studied. Most research has focused on prokaryotes, whereas microscopic fungi, which have high biotechnological potential in biodegradation processes, remain unexplored in this context.

Rhodotorula are unicellular fungi characterized by a typical yeast cell structure, the ability to produce pigments, and the absence of true mycelium (Cordeiro, 2019). Representatives of the genus *Rhodotorula* exhibit high tolerance to adverse environmental conditions, including the presence of xenobiotics and anthropogenic toxicants. Due to their metabolic flexibility, these yeasts are capable of surviving and functioning in aggressive ecological niches, which makes them of interest as potential agents in microbial pollutant degradation technologies. When analyzing the biological characteristics of the studied species, it is important to note certain features – slightly different colony characteristics formed on solid nutrient media, pigment production intensity, the ability to utilize

carbon and nitrogen sources, optimal temperature regime for growth, and stress tolerance.

The experience of using *Rhodotorula* spp. in environmental bioremediation from other harmful compounds – such as organochlorine pesticides, surfactants, and heavy metals – indicates the presence of the necessary enzymatic systems. Regarding N-(phosphonomethyl)glycine, this capability remains the subject of scientific analysis, but existing data already point to the promise of further research in this direction (Cordeiro, 2019; Ruffolo et al, 2023; Feng et al, 2020; Mohy-Ud-Din et al, 2023).

Therefore, the aim of our work was to study the long-term effect of N-phosphonomethylglycine on selected indicators of the pro- and antioxidant status of *R. minuta* and *R. rubra*.

Materials and Methods. The material for our study consisted of pure cultures of microorganisms *Rhodotorula rubra* and *Rhodotorula minuta*, kindly provided by the D.K. Zabolotny Institute of Microbiology and Virology.

Carotenoid-producing yeasts were cultivated on Sabouraud medium. The incubation of inoculum was carried out at a temperature of 28 °C for 48 hours with appropriate aeration (100 rpm).

The long-term effect of N-(phosphonomethyl)glycine on growth parameters and selected biochemical indicators was analyzed in a series of laboratory experiments using three working concentrations of the herbicide: 25, 75, and 125 mg/L. Cultivation was carried out in 250 ml Erlenmeyer conical flasks, with a fermentation working volume of 50 ml and a standardized inoculum volume of 10% under similar conditions.

Growth dynamics were assessed by measuring the optical density of the culture fluid daily by photometry at a wavelength of 540 nm. To determine the effect of N-(phosphonomethyl)glycine (glyphosate) on yeast cell viability, the number of colony-forming units (CFU) was counted on solid nutrient medium.

At the end of the experiment, the cell biomass was separated by centrifugation, and cell degradation was performed using ultrasound (7200 μA).

The intensity of lipid peroxidation (LPO) was assessed by the content of thiobarbituric acid-reactive substances (TBARS), which are secondary metabolites of oxidative lipid damage. The method is based on the formation of a chromophore complex between aldehyde derivatives (mainly malondialdehyde) and thiobarbituric acid (Sehin, 2016).

Protein content was determined by the Lowry method (Waterborg and Matthews, 1984).

For the quantitative and qualitative assessment of the carotenoid complex, the acetone extraction method was used followed by spectrophotometric analysis at wavelengths of 450 nm, 509 nm, and 537 nm. Carotenoid content was calculated according to formulas (Biehler et al, 2010).

The results of the experimental data were processed statistically using software Microsoft Excel. At the same time, the results were reliable at the level of reliability $p \leq 0,05$ according to the Student's criterion.

Results and Discussion. Under current conditions of widespread use of glyphosate-based herbicides in agriculture, there is a growing need for a detailed study of their effects on non-phytobiotic components of ecosystems, particularly soil microorganisms.

Yeasts of the genus *Rhodotorula*, particularly *R. minuta* and *R. rubra*, play an important role in biogeochemical cycles, participating in the transformation of organic substances, synthesis of biologically active compounds, and maintenance of microbial diversity. Studying their sensitivity and adaptive response to glyphosate exposure is relevant both in the context of assessing ecotoxicological risks and for the development of biotechnological approaches to the bioremediation of contaminated soils.

The assessment of the long-term impact of different concentrations of glyphosate on the development of the *R. rubra* culture showed both stimulatory and inhibitory effects depending on the pollutant dose and exposure duration. With the application of glyphosate at a concentration of 25 mg/L, a slight «accumulation» of culture biomass was observed. As the concentration increased to 75 mg/L, the optical density of the culture decreased by the 4th day of the experiment, which indicated the beginning of growth inhibition of the culture under the influence of the herbicide.

The most pronounced toxic effect was recorded at a concentration of 125 mg/L, when the optical density decreased by 1,3 times, indicating significant inhibition of *R. rubra* growth.

The analysis of glyphosate's effect on the optical density of the *R. minuta* culture revealed trends similar to the previous species, but with a less pronounced response to low doses.

It is known that different types of microorganisms respond differently to glyphosate: in some cases, a reduction in fungal biomass and species diversity is observed, while other organisms exhibit adaptive properties or grow more actively in response to low doses of the herbicide (Kourtaki et al, 2025). In our opinion, the results of long-term glyphosate exposure may be due to several factors. First of all, the species-specific characteristics of the studied yeasts should be taken into account. It is

known that the composition and structure of the cell wall (content of chitin, β-glucans, mannans) can influence the cells' ability to bind toxicants, including glyphosate, thereby reducing its penetration into the cell. Additionally, the herbicide concentration is an important factor that determines the nature of its effects. At low doses, glyphosate can serve as a source of available carbon or phosphorus, temporarily stimulating the growth and metabolic activity of microorganisms. However, at medium and high concentrations, glyphosate exhibits cytotoxic effects associated with the inhibition of EPSPPS enzyme in the cells of microscopic fungi, which disrupts the synthesis of aromatic amino acids. Finally, the duration of exposure is also significant. In the early days, activation of adaptive responses is possible. However, over 14 days, the effect of the toxicant may accumulate, leading to a decrease in the effectiveness of defense mechanisms.

The number of colony-forming units (CFU) is one of the key indicators of microorganism viability and allows the assessment of cells' ability to reproduce after exposure to a certain factor, including stress — chemical, physical, or biological. On the 14th day of the experiment, the following was recorded: in the control variants, the average CFU count of *R. rubra* was $5{,}3\times10^4$ CFU/mL, and *R. minuta* – $4{,}9\times10^4$ CFU/mL; at a concentration of 25 mg/L, both strains did not show a significant increase in numbers: for *R. rubra* – up to $5{,}8\times10^4$ CFU/mL, and for *R. minuta* – up to $5{,}2\times10^4$ CFU/mL.

Further increase in glyphosate concentration led to a gradual decrease in the number of viable cells. At 75 mg/L, the CFU count decreased on average by 20%, and the greatest growth inhibition was observed at a concentration of 125 mg/L: the number of viable *R. rubra* cells was only $2{,}7\times10^4$ CFU/mL (a decrease of almost 2 times), and *R. minuta* – down to $2{,}2\times10^4$ CFU/mL.

Thus, the effect of glyphosate on the colony-forming ability of yeasts is dose-dependent. In our opinion, one possible explanation for the observed increase in yeast CFU in the presence of a low concentration of glyphosate (25 mg/L) is the manifestation of a hormetic effect. It is known that low doses of some toxic substances may exert a stimulatory effect on cells due to the activation of defense systems and mobilization of metabolic reserves. Such mild stress exposure potentially stimulates initial colony formation processes, particularly yeast cell proliferation and growth. On the other hand, colony growth inhibition at high glyphosate concentrations (75 and 125 mg/L) may be caused by its toxic action, which is realized through the inhibition of enzymatic systems necessary for amino acid biosynthesis or through the induction of oxidative stress. To verify the latter, the content of lipid peroxidation products and the level of carotenoids – low-molecular-weight antioxidants – were determined.

After 14 days of exposure, a dose-dependent increase in the level of TBARS was observed in the yeasts *R. rubra* and *R. minuta*, indicating an intensification of lipid peroxidation processes (Fig. 1).

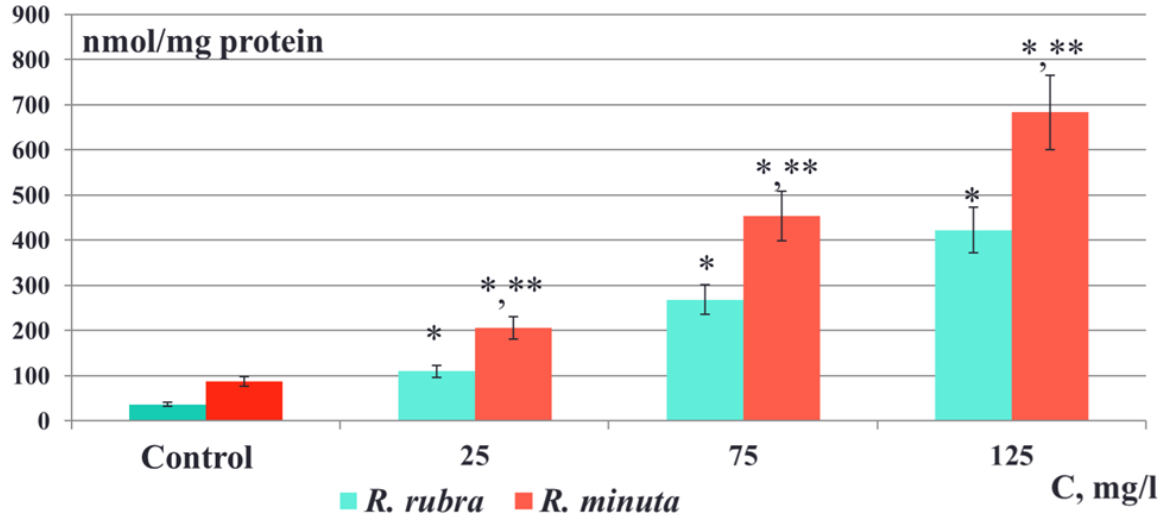

Fig. 1. TBARS content in Rhodotorula spp. cells after 14-day exposure to N-(phosphonomethyl)glycine

Note (here and hereafter):

** – statistically significant difference compared to the control (p≤0,05)*

*** – statistically significant difference between the two species (p≤0,05)*

a – changes in R. rubra culture density

b – changes in R. minuta culture density

The increased glyphosate content in the *Rhodotorula* cultivation medium clearly causes severe damage to membrane structures, as confirmed by the progressive rise in TBARS levels, with a maximum recorded at 125 mg/L of the pesticide.

Overall, *R. minuta* demonstrates a higher level of TBARS in all experimental variants, indicating its lower ability to withstand oxidative stress compared to *R. rubra*, which exhibits greater tolerance to the action of the herbicide.

Under the influence of glyphosate in *Rhodotorula* cells, redox balance is evidently disrupted. This is manifested in the increased formation of reactive oxygen species (ROS), leading to oxidative stress. In response, the antioxidant defense system is activated, which includes enzymes as well as non-enzymatic antioxidants (Cordeiro, 2019; Dayan et al, 2015; Kőmíves and Schröder, 2016).

As observed by us (Fig. 2), glyphosate at concentrations of 25 and 75 mg/L did not lead to statistically significant changes in the level of β-carotene activity in either of the studied microorganisms, but it did stimulate the production of genus-specific carotenoids: under the influence of 75 mg/L, torulene concentration increased on average by 1,8 times, and torularodin – by 1,5 times.

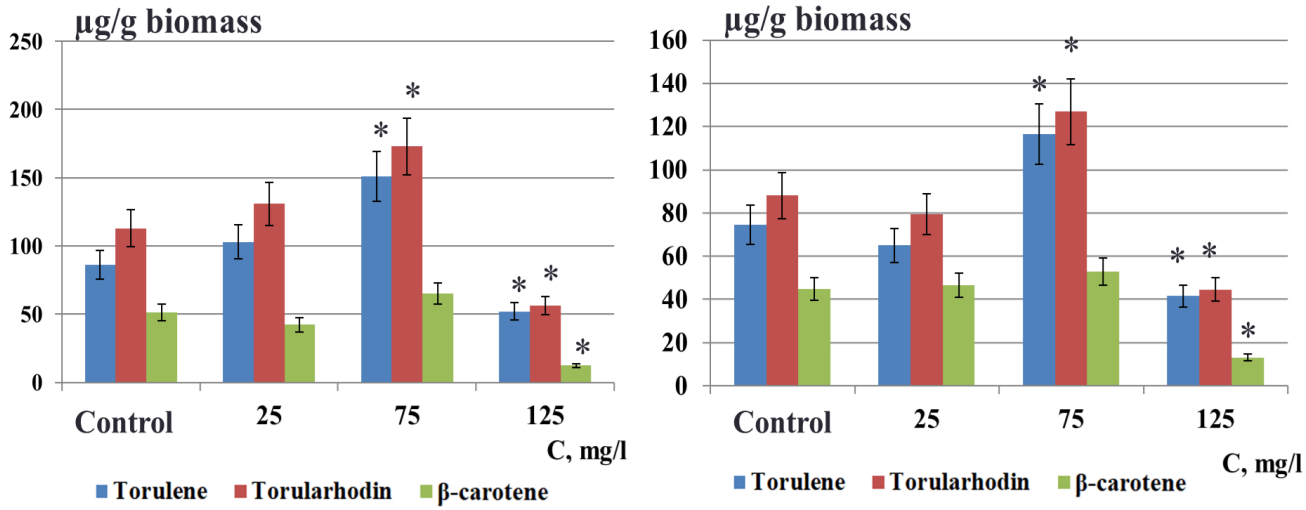

Fig. 2. Content of total and genus-specific carotenoids in** Rhodotorula **spp. cells under glyphosate exposure after 14-day incubation

Note: a – R. rubra, b – R. minuta

At the highest concentration of glyphosate, however, a significant decrease in the levels of all studied pigments was observed. The torulene content decreased by nearly 40%, torularodin was reduced by half, and β-carotene showed the most substantial decline. The drop in torularodin content was particularly striking. It is known that torularodin is an oxidized analog of torulene – it contains a carboxyl group (-COOH), which gives it slight polarity and allows it to localize in more hydrophilic regions of the cell envelope. Torularodin has an absorption spectrum similar to that of β-carotene, but its antioxidant capacity is higher due to the presence of a functional group that interacts more actively with free radicals. This pigment is a key protective factor for *Rhodotorula* cells when exposed to toxicants, UV radiation, heavy metals, and other stressors. It neutralizes the superoxide anion ($O_2^{-}\cdot$) and hydrogen peroxide (H_2O_2), and also maintains redox balance (Marcelino et al, 2020).

Since carotenoids play a role in protection against oxidative stress, the disruption of their synthesis under the influence of glyphosate may lead to increased sensitivity of cells to adverse conditions. It is known that in *R. rubra*, the biosynthesis of torulene and β-carotene is closely related to mitochondrial function and requires NADPH. In the presence of glyphosate, researchers suggest that there is a shift in redox balance and a reduction in the availability of cofactors, which lowers pigment synthesis. In experiments involving *R. glutinis*, a species closely related to *R. rubra*, the addition of 169 mg/L of glyphosate to the medium reduced pigment concentrations by nearly half, and the colony color changed from deep red to pale pink (Hashem et al, 2018; Cheng et al, 2016; Gong et al, 2018).

Conclusions. The nature of glyphosate's action is dose- and time-dependent, meaning it is determined by both the concentration of the substance and the duration of exposure. N-(phosphonomethyl)glycine exerted an inhibitory effect on the development of carotenoid-producing yeasts only under long-term exposure at the highest concentration, inducing oxidative stress accompanied by the accumulation of lipid peroxidation products, a decrease in carotenoid levels, and an overall inhibitory effect on culture density and colony-forming activity.

***Conflict of interest:** The authors declare that the study was conducted in the absence of any*

commercial or financial relationships that could be interpreted as a potential conflict of interest.

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ДОВГОТРИВАЛИЙ ВПЛИВ N-(ФОСФОНОМЕТИЛ)ГЛІЦИНУ НА РОЗВИТОК RHODOTORULA SPP.

A.V. Tykhenka, L.M. Vasina

Чернівецький національний університет імені Юрія Федьковича
вул. Коцюбинського, 2, Чернівці, 58012
e-mail: l.vasina@chnu.edu.ua

Боротьба з інвазивними видами рослин у агроекосистемах здебільшого повязана із застосуванням синтетичних гербіцидів через їх високу ефективність. Одним з найбільш вживаних є гліфосат [N-(фосфонометил)гліцин]. Це неселективний системний гербіцид широкого спектру дії, що застосовується для боротьби з однорічними та багаторічними рослинами. Ця сполука діє шляхом інгібування ферменту 5-енолпірувілшикімат-3-фосфатсинтази, що бере ключову участь у шикімантному шляху та відповідає за синтез проміжного продукту в біосинтезі ароматичних амінокислот. N-(фосфонометил)гліцин демонструє неоднозначний вплив на ґрунтові спільноти мікроорганізмів. Одні концентрації гербіциду здатні стимулювати мікробну активність, збільшуючи різноманітність і складність мережі. В інших випадках – гербіцид виявляє негативну дію на виживаність та структуру ґрунтових мікроорганізмів. Мало відомим залишається вплив гербіциду на мікроскопічні гриби, зокрема представників роду Rhodotorula, що характеризуються широким розповсюдженням у різноманітніх екологічних нішах, високою метаболічною активністю (синтез пігментів, ферментів, екзополісахаридів, ергостеролу), можливістю існування у мікробних консорціумах, здатністю до адсорбції.

У роботі досліджували вплив гербіциду N-(фосфонометил)гліцину на фізіолого-біохімічні показники двох видів дріжджів: R. rubra та R. minuta. У ході роботи визначено зміну ростових характеристик за довготривалого впливу гербіциду, вміст каротиноїдів, рівня тіобарбітурат-активних речовин.

Встановлено, що N-(фосфонометил)гліцин у вищих з досліджуваних концентрацій виявляв дозо- та часозалежним інгібуючий вплив на розвиток каротиногенних дріжджів, індукуючи при цьому розвиток оксидативного стресу, що супроводжувався накопиченням продуктів пероксидного окиснення ліпідів і зміною

рівня основним каротиноїдів. При цьому відзначалося зниження щільності культури обох досліджуваних видів, зменшення кількості планктонних колонієутворювальних одиниць. R. minuta відрізнялася більшою чутливістю до дії полютанта, що підтверджувалося кардинальнішими змінами досліджуваних показників – значним зменшенням кількості життєздатних клітин, кратним зростанням рівня кінцевих продуктів пероксидного окислення ліпідів, зниженням рівня каротиноїдів.

Ключові слова: N-(фосфонометил)гліцин, Rhodotorula spp., фунгітоксичність, дозозалежність.

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ORCID ID
Liliia Vasina: https://orcid.org/0000-0001-5458-3537