PHISICOCHEMICAL BIOLOGY

Original article
DOI: https://doi.org/10.21285/2227-2925-2022-12-3-394-405

Influence of endophytic and epiphytic nitrogen-fixing bacteria on the content of negative allelopathic compounds in root exudates of pea (*Pisum sativum* L.) seedlings

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Abstract. *Substances that have a harmful effect on living organisms include N-phenyl-2-naphthalamine and phthalates, which are synthesized and widely used in the chemical industry. At the same time, N-phenyl-2-naphthylamine was found in the aerial parts and in the roots of some plant species, phthalates were found in many plant species and in bacteria. The aim of this research was to study the protective (antimicrobial) reaction of pea (Pisum sativum* L.*) seedlings of the Torsdag variety to the inoculation with bacteria Rhizobium leguminosarum* bv. *viceae (endosymbiont) and Azotobacter chroococcum (ectosymbiont) introduced into the aqueous medium of root growth were studied. Changes in the content of negative allelopathic compounds (pisatin, N-phenyl-2-naphthylamine, phthalates) in root exudates were the reaction indicators. After the inoculation, the seedlings grew for 24 h in the BINDER KBW-240 chamber at 21 °C, with lighting of 81 μM.m^{-2}·sec^{-1} and a 16/8 h day/night photoperiod. In ethyl acetate extracts from the aqueous medium where the seedling roots were immersed, the content of the compounds was determined by HPLC, while changes in the composition and ratio of phthalates were determined by GC-MS. Data indicating the different ability of both bacterial species to degrade N-phenyl-2-naphthylamine to phthalates and the dependence of this process activity in the bacteria studied on its concentration in the medium were presented. N-phenyl-2-naphthylamine differently but negatively affected the viability and growth of the bacteria used in the experiments. A different effect of rhizobia and azotobacter on the content of the above named compounds and on the ratio of types of phthalates in root exudates was elicited.*

Keywords: *Pisum sativum* L.*, root exudates, Rhizobium, Azotobacter, pisatin, N-phenyl-2-naphthylamine, phthalates*

Acknowledgements. *The authors express their deep gratitude to the research member of Siberian Institute of Plant Physiology and Biochemistry SB RAS, PhD A. S. Moritz, I. S. Kapustina for his technical assistance. Analytical work was performed with the equipment of the Common Use Center "Bioanalytics" of Siberian Institute of Plant Physiology and Biochemistry SB RAS (Irkutsk).*

For citation: Makarova L. E., Petrova I. G., Sokolova N. A., Makarov S. S., Pionkevich V. A. Influence of endophytic and epiphytic nitrogen-fixing bacteria on the content of negative allelopathic compounds in root exudates of pea (*Pisum sativum* L.) seedlings. *Izvestiya Vuzov. Prikladnaya Khimiya i Biotekhnologiya = Proceedings of Universities. Applied Chemistry and Biotechnology.* 2022;12(3):394-405. https://doi.org/10.21285/2227-2925-2022-12-3-394-405.

ФИЗИКО-ХИМИЧЕСКАЯ БИОЛОГИЯ

Научная статья
УДК 581.5:633. 358:577.13

Влияние эндофитных и эпифитных азотфиксирующих бактерий на содержание негативных аллелопатических соединений в корневых экссудатах проростков гороха *(Pisum sativum* L.)

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Аннотация. *К веществам, оказывающим вредное воздействие на живые организмы, относятся N-фенил-2-нафтиламин и фталаты, которые синтезируются и широко используются в химической промышленности. В то же время N-фенил-2-нафтиламин был обнаружен в надземных частях и в корнях некоторых видов растений, фталаты были найдены во многих видах растений и в бактериях. Исследование направлено на изучение защитной (антимикробной) реакции проростков гороха (Pisum sativum* L.*) сорта Торсдаг на инокуляцию бактериями Rhizobium leguminosarum* bv. *viceae (эндосимбионт) и Azotobacter chroococcum (эктосимбионт), вносимыми в водную среду роста корней. Показателями реакции были изменения содержания негативных аллелопатических соединений (пизатин, N-фенил-2-нафтиламин, фталаты) в корневых экссудатах. После инокуляции проростки росли одни сутки в камере BINDER KBW-240 при температуре 21 ºС, освещении 81 μМ.м$^{-2}$·с$^{-1}$ и фотопериоде 16/8 ч (день/ночь). В этилацетатных экстрактах из водной среды, куда погружали корни проростков, содержание соединений определяли методом ВЭЖХ, а изменения в составе и соотношении фталатов – методом ГХ-МС. Установлено различное влияние ризобий и азотобактера на содержание вышеперечисленных соединений и на соотношения видов фталатов в корневых экссудатах. Представлены данные, указывающие на разную способность обоих видов бактерий деградировать N-фенил-2-нафтиламин до фталатов и на зависимость активности данного процесса у исследуемых бактерий от его концентрации в среде. N-фенил-2-нафтиламин по-разному, но негативно влиял на жизнеспособность и рост использованных в экспериментах бактерий.*

Ключевые слова: *Pisum sativum* L.*, корневые экссудаты, Rhizobium, Azotobacter, пизатин, N-фенил-2-нафтиламин, фталаты*

Благодарности. *Авторы выражают глубокую признательность научному сотруднику Сибирского института физиологии и биохимии растений СО РАН, к.т.н. А. С. Морицу, а также И. С. Капустину за техническую помощь. Аналитическая работа выполнена на оборудовании ЦКП «Биоаналитика» Сибирского института физиологии и биохимии растений СО РАН (г. Иркутск).*

Для цитирования: Макарова Л. Е., Петрова И. Г., Соколова Н. А., Макаров С. С., Пионкевич В. А. Влияние эндофитных и эпифитных азотфиксирующих бактерий на содержание негативных аллелопатических соединений в корневых экссудатах проростков гороха (*Pisum sativum* L.) // Известия вузов. Прикладная химия и биотехнология. 2022. Т. 12. N 3. С. 394–405. (In English). https://doi.org/10.21285/2227-2925-2022-12-3-394-405.

INTRODUCTION

N-phenyl-2-naphthylamine and phthalates are well known compounds, synthesized in chemical industry. These chemicals are known to be toxic for living organisms. At the same time N-phenyl-2-naphthylamine was found in plants [1–4], phthalates were found both in plants and bacteria [4, 5].

N-phenyl-2-naphthylamine and phthalates can inhibit bacterial growth [6, 7], apparently similar to phytoalexins [8]. At the same time, bacteria can catabolize N-phenyl-2-naphthylamine with the formation of phthalates [6, 9], which, probably, with the predominant formation of phthalates of negative action by bacteria of certain species, is important in the competitive interaction between bacteria of the soil microbiome [10]. The foregoing suggests that N-phenyl-2-naphthylamine and phthalates found in the root exudates of legumes [4] act as regulators of the bac-

terial concentration and composition in the root zone of these plants. Changes in the content of these compounds in root exudates may be due to the effect of biotic and abiotic factors on plants [10].

Soil microorganisms, including intracellular, endophytic and epiphytic nitrogen fixers, are known to play a special role in supplying plants with nitrogen [11]. Bacteria that settle in root and stem nodules developing in plants are characterized by higher nitrogen fixation efficiency. It is believed that rhizospheric free-living nitrogen-fixing bacteria genera *Azotobacter*, *Azospirillum*, and *Bacillus* serve as possible suppliers of nitrogen compounds available for a legume plant that are necessary at early stages of the formation of nodule nitrogen-fixing structures [12].

According to the research results [13], the combined effect of bacteria genera *Rhizobium* and *Azotobacter* on a legume plant is useful for its growth. It was shown that the free-living bacteria *Azotobacter chroococcum*, in common with rhizobia, can cause protective reactions in root cells of legume plants in the initial periods after inoculation. The reactions can be even more noticeable than nodule bacteria.

Protective reactions of plants in response to the introduction of infection appear not only at the level of cells of their organs. They also appear via the excretion of components with antimicrobial effects by their root cells into the root zone. The substances of acts of that nature include compounds with different structures. First of all, they include phytoalexins, which differ in chemical structure not only in different plant genera, but also in species of the same genus [14]. These compounds are intensely synthesized by plants not only when phytopathogens act on them. They are also intensely synthesized when bacteria entering into a mutualistic relationship with the plant act on them. Biotic stresses are one more reason for these compounds to be synthesized intensely [8, 15]. At high concentrations, a number of low-molecular-weight compounds synthesized by plants can also have antimicrobial effects. These compounds are called phytoanticipins [16]. Probably, phytoanticipins are aromatic compounds found in plant cells in their normal living conditions; they are highly accumulated in plant tissues under stress and are secreted by the plant into exudates. Cinnamyl phenols, 2′-methoxychalcone and isoflavonoids, which were found in tissues of pea plants under stress conditions, can be reckoned among such compounds [17]. Along with the phytoalexin pisatin, N-phenyl-2-naphthylamine and phthalates found in roots and root exudates of legume plants can be named among the compounds, which are synthesized in pea and other legume plants, and which have negative effects on soil microflora [4]. Currently, N-phenyl-2-naphthylamine and phthalates are considered as substances dangerous to living organisms [18, 19]. The negative role of pisatin was also shown for certain plant species [20].

As a result of the set of our experiments [10], the dependence of the content of pisatin, N-phenyl-2-naphthylamine, and dibutyl phthalate in the root exudates of pea seedlings on the size of the root and on the type of effect on the roots by the bacteria have been studied. The influence of lighting conditions and temperature on the amount of N-phenyl-2-naphthylamine and phthalates, and the influence of temperature conditions on the composition of phthalates have been observed.

When considering the significance of the above compounds in the interaction between macro- and micropartners, their role as negative regulators of bacterial growth should be the first thing to be taken into account. As it was noted above, N-phenyl-2-naphthylamine and phthalates can inhibit the growth of bacteria entering into symbiotic relationships with pea plants. In the issue of the relationship of a legume plant with free-living nitrogen-fixing bacteria that do not penetrate its tissue, the participation of these compounds has not been studied yet. It is our belief that N-phenyl-2-naphthylamine and phthalates, together with pisatin, are potential candidates for controlling the growth of associative bacteria similarly, which can accumulate in the root zone of a pea plant together with endophytic bacteria.

The well-known characteristic of rhizobacteria (the ability to catabolize many aromatic compounds) can be important for determining the role of these compounds in relation to the bacterial microflora. That is, along with aromatic compounds secreted into the environment by the plant, their biodegradation products can well turn out to be modulation factors for metabolic processes in bacterial cells. In terms of determining the mechanism of the negative effect of one of the components of root exudates of legume plants N-phenyl-2-naphthylamine on bacteria, the feature of some species of soil bacteria to degrade it to phthalates and actively secrete the latter to the environment may cause interest [6, 7]. Thus bacteria can contribute to changes in the concentration of N-phenyl-2-naphthylamine and the quantitative ratio in the composition of phthalates in the root zone of a plant.

The aim of this research was to compare the protective (antimicrobial) reaction of pea seedlings, which appears at the rhizosphere level in the initial periods of infection of their roots with nitrogen-fixing bacteria *R. leguminosarum* bv. *viceae* and *Azotobacter chrococcum*, different in their specificity of relations with pea plants (endosymbiont-mutualist and free-living bacteria). For this purpose, changes in the content of probable negative regulators of reproduction of both bacterial species (pisatin, N-phenyl-2-naphthylamine, and phthalates) were studied in the root exudates of pea seedlings 24 h after the inoculation with the bacteria.

EXPERIMENTAL SECTION

Research objects. Pea (*Pisum sativum* L.) seedlings of the Torsdag variety and bacteria *R. legumi-nosarum* bv. *viceae* (strain RCAM1022, obtained from the All-Russian Research Institute of Agricultural Microbiology, Pushkin, Russia) and *Azotobacter chroococcum* (strain Az dl0 VKM B-2272 D from the collection of the Collective Use Center "Bioresource Center" of Siberian Institute of Plant Physiology and Biochemistry, Siberian Branch of the Russian Academy of Sciences (Irkutsk, Russia) were the research objects.

Plant material. Pea seeds were decontaminated by washing with tap water and soap, followed by treatment with a 3% hydrogen peroxide solution. Seed germination and seedling growth took place for 48 h on sterile filter paper moistened with tap water, in a thermostat at 21 °C, without lighting. Further growth of seedlings took place in the BINDER KBW-240 chamber at 21 °C, with lighting of 81 $\mu M.m^{-2}.sec^{-1}$, and a 16/8 h photoperiod (day/night) for 24 h. It was performed after immersing the roots of the seedlings in aqueous solutions containing microelements [10]; the solutions were poured into glass vessels with the outer surface darkened with a light-tight film. The volume of the solution in the vessels was 250 ml; the number of seedlings per 1 vessel was 36. The average sizes of the roots of the initial seedlings were 35–38 mm in length; at the end of the experiment they were 45–50 mm long. To inoculate plants, the bacterial culture was introduced into the aqueous medium in the form of an aqueous wash off from a solid agar medium to a concentration of 4.0×10^5 cells/ml. The inoculation was carried out simultaneously with the placement of the roots of the seedlings in the aqueous medium. Same-aged uninoculated seedlings served as control.

Cultures of bacteria. When growing on solid agarized media, they were prepared according to the previously accepted scheme [4] for nodule bacteria; they were prepared according to Ashby for associative bacteria. Planktonic cultures of bacteria used to obtain growth indicators and to study the degradation products of N-phenyl-2-naphthylamine were grown in a minimal liquid medium. The medium was prepared in the way described in the research, but glucose was used as a carbohydrate food source for bacteria [6]. After the preliminary 24 h adaptation in the indicated medium, the bacteria were grown in the presence of 10 or 100 μM N-phenyl-2-naphthylamine (commercial preparation, Sigma, USA) for 24–48 h in flasks in a rotatory shaker at 20–22 °C in the dark. The bacterial titer in the media was 1.5×103 cell/mL at the start of the experiments in which N-PNA degradation was analyzed; it was 4.5×10^3 cells/mL at the start of the experiments studying the effects of N-phenyl-2-naphthylamine on the bacterial growth. The bacterial titer was determined by the optical density of the bacteria-containing media measured in the Immunochem-2100 plate spectrophotometer at 675 nm (High Technology Inc., United States).

Study of the effect of N-phenyl-2-naphthylamine on the growth of the bacteria. The growth of the bacteria in the presence of 10 or 100 μM N-phenyl-2-naphthylamine, which was carried out in flasks with a minimal liquid medium, prepared according to [21] was controlled by the colonies of forming units (CFUs) indices. To calculate the number of CFUs, a bacterial suspension from the 1- and 2-day culture media was plated onto solid agarized media, the preparation methods of which are named above.

The bacterial titer in the media was 4.5×10^3 cells/mL at the start of the experiments studying the effects on the bacterial growth. The bacterial titer was determined by the optical density of bacterial-containing media, as described above.

Preparation of extracts with allelopathic substances from root exudates and culture media. Phenolic compounds of the root exudates were extracted from the aqueous medium (into which the roots of the seedlings had been previously immersed) after the acidification with HCl solution to pH 3.0. Three-stage extraction was carried out using ethyl acetate (1:1, v/v), which was then evaporated with a cold air stream in the dark. The dry residue was dissolved in 1.0 ml of methanol. The compounds contained in the methanol extract were studied by HPLC. After that, the 0.8 ml from remaining part of the same extract was evaporated in a vacuum until the removal of methanol, the residue was re-dissolved into ethyl acetate and after silylation (BSA + GMDS) by GC-MS analysis, the composition of phthalates was studied in it.

Before the extracts from the culture media containing aromatic products of N-phenyl-2-naphthylamine degradation were obtained, the grown culture was centrifuged at 8000 g for 20 min at 4 °C using a centrifuge of the Avanti J-26 XP JLA model (Beckman Coulter, USA). The aromatic compounds were extracted from the supernatant with ethyl acetate in the way described above. The obtained extracts were vacuum-evaporated in the dark to small volumes for conducting GC-MS analysis of the compounds present in them without prior silylation. In all cases, extracts (methanol and ethyl acetate) were placed in glass vials.

Determination of the content of allelopathic compounds by HPLC. The content of pisatin, N-phenyl-2-naphthylamine, and dibutyl phthalate was determined by HPLC using the Shimadzu LC-10ATvp chromatograph with a UV detector (Shimadzu, Japan). The compounds in the adsorption profiles were identified by the retention time of taps, which was confirmed by UV spectra obtained in a stopped eluent flow for taps and for the substances under study. Authentic samples of N-phenyl-2-naphthylamine

(Sigma, USA), dibutyl phthalate (Reachim, Russia), and pisatin, which had been kindly provided earlier by Prof. H. D. VanEtten (Department of Plant Sciences, University of Arizona,United States), were used to identify and construct calibration curves. The phenolic components contained in the extracts were separated on a Perfect Bond column (250×4.6 mm, 5 μm) (MZ Analisentechnik, Germany) in an increasing A:B gradient from 30 to 90% for 80 min at a rate of 0.5 mL/min (A–acetonitrile and B–0.2 N Li perchlorate in 0.1% aqueous solution of trifluoroacetic acid, pH 4.0). The compounds were detected at 280 nm. The calculations were based on adsorption profiles of peak heights with the calibration curves constructed for different N-PNA concentrations of the compounds studied. R-squared values (R^2) were: 0.96 for N-phenyl-2-naphthylamine, 0.98 for dibutyl phthalate, 0.99 for ethyl phtalate and 0.99 for pisatin.

Study of the composition of aromatic compounds in extracts by GC-MS analysis. The composition of aromatic compounds in extracts was analyzed in the 7000 QQQ Triple Quad/7890A MSD/DS GC/MS (Agilent Technology, USA). The injected sample volume was 0.2 μL. The evaporator temperature was 250 °C, the ion source temperature was 230 °C, the detector temperature was 150 °C, and the temperature of the transfer line between the gas chromatograph and mass spectrometer was 280 °C. The scanning range was between 50 and 600 Da. The capillary column was HP-5MS (30 m×0.250 mm×0.50 μm); the stationary phase was (5% phenyl) methylpolysiloxane. The temperature gradient was from 100 to 280 °C at the rate of 5 °C/min, 2 min at 280 °C, and then from 280 to 340 °C at the rate of 5 °C/min. The mobile phase was helium, and the gas-flow rate was 1 mL/min. The current flow division mode was 5:1. The electron ionization mode had ionization energy of 70 eV. The analysis was performed in total ion current registration mode (SCAN). The NIST08 and WILEY7 mass-spectra libraries, along with the comparison with the authentic dibutyl phthalate (Reakhim, Russia), bis(2-ethylhexyl) phthalate (synonymous to dioctyl phthalate), and diethyl phthalate (Sigma Aldrich, Germany) samples, were used to identify the analyzed compounds.

Statistics. The results obtained were statistically processed; the Figures and Tables show the mean values and their standard deviations obtained from three independent experiments.

RESULTS AND DISCUSSION

When comparing the data obtained by HPLC for the control plants and ones inoculated with *R. leguminosarum* bv. *viceae* and *Azotobacter chroococcum*, a different character of the effect of the two bacterial species on the content of pisatin, N-phenyl-2-naphthylamine, diethyl phthalate and dibutyl phthalate in the root growth medium is observed (Fig. 1). The inoculation with *Rhizobium* contributed to an increase in the total content of the above listed negative allelopathic compounds; it happened mainly due to pisatin. As a result of the inoculation with *Azotobacter*, the content of N-phenyl-2-naphthylamine decreased by more than 10 times; the content of pisatin and dibutyl phthalate decreased by 1.2 and 1.4 times, respectively. In summation, the total number of these compounds per 1 seedling in the aqueous medium of root growth in the presence of rhizobia was 2.3 times higher than in the presence of azotobacter.

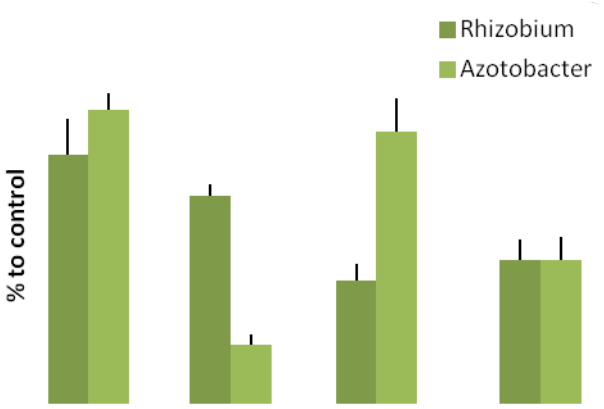

Fig. 1. Changes in the content of pisatin, N-phenyl-2-naphthylamine (N-PNA), diethyl phthalate (DEP) and dibutyl phthalate (DBP) in the pea seedling root growth medium after the addition of *Rhizobium leguminosarum* bv. *viceae* (Rhizobium), *Azotobacter chroococcum* (Azotobacter). Y-axis: % to control (control – seedlings not inoculated with bacteria)

Рис. 1. Изменение содержания пизатина, N-фенил-2-нафтиламина (N-PNA), диэтилфталата (DEP), дибутилфталата (DBP) в среде роста корней проростков гороха после внесения в нее *Rhizobium leguminosarum* bv. *viceae* (Rhizobium), *Azotobacter chroococcum* (Azotobacter). По оси ординат: % к контролю (контроль – неинокулированные бактериями проростки)

Four types of phthalates were identified in the root exudates of pea seedlings by GC-MS analysis: dibutyl phthalate, dioctyl phthalate, diethyl phthalate and butyl tetradecyl phthalate (Fig. 2).

According to the percentage of peak area values, dibutyl phthalate and dioctyl phthalate were almost equally dominant in the control plants and in those inoculated with rhizobia in their root growth medium. When inoculated with azotobacter, dioctyl phthalate predominated among the phthalates: its peak area was almost 2 times higher than those for dibutyl phthalate and diethyl phthalate. In plants of all growing options, the content of butyl-tetradecyl phthalate (an intermediate compound in the formation of diphthalates with the same hydrocarbon chains) was 3–6%.

The inoculation with *Rhizobium* was mentioned above to contribute to an increase in the total content of negative allelopathic compounds in the root growth

medium, which happened mainly due to pisatin (Fig. 1). This phytoalexin is synthesized in pea plant cells. Among the compounds analyzed, it is most studied by its antifungal and antibacterial characteristics, which are important for inter-relationships amongst organisms. In pea plants, increased accumulation of pisatin was observed under the influence of fungal and bacterial infections, as well as under the conditions of abiotic stress [8, 15, 16].

Pisatin can suppress bacteria genus *Rhizobium* that nodulate roots of legume plants [8, 22]. Different strains of *Rhizobium leguminosarum* show uneven susceptibility to pisatin. A decrease in susceptibility to pisatin in some representatives of fungal pathogens of legumes genus *Fusarium* was associated with their ability to degrade this compound [16]. Similar phenomena of pisatin degradation in bacteria have not been noted in the scientific literature, but there are some reasons to believe their existence in these microorganisms [22]. Whithin our research, a decrease in pisatin level in the growth medium of the roots of pea seedlings, which arose under the influence of *Azotobacter chroococcum*, indirectly indicates its degradation by bacteria, as compared with the control plants (Fig. 1).

As it was said above, when inoculated with *Azotobacter*, the total content of the studied substances with a negative effect in the root growth medium decreased mainly due to N-phenyl-2-naphthylamine. In case of the inoculation with *Rhizobium* and *Azotobacter,* we bind the difference in the content of this compound in the composition of root exudates to the uneven activity of this compound degradation in the cells of the same species of bacteria. We believe that, when inoculated with *Azotobacter,* a decrease in the content of N-phenyl-2-naphthylamine in the growth

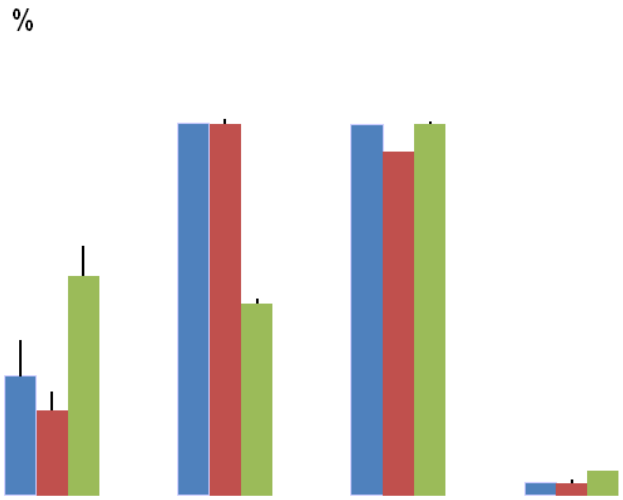

Fig. 2. The effect of bacteria on the ratio of phthalates in the root exudates of pea seedlings 1 day after inoculation. 1 – diethyl phthalate; 2 – dibutyl phthalate; 3 – dioctyl phthalate; 4 – butyl tetradecyl phthalate; Kontrol – without bacteria, + *Rhizobium* and + *Azotobacter* – introduction of bacteria *R. leguminosarum* bv. *viceae* and *Azotobacter chroococcum*, respectively; S, % – relative area indicator for peaks in the chromatogram (GC-MS) for phthalates

Рис. 2. Влияние бактерий на соотношение фталатов в корневых экссудатах через одни сутки после инокуляции. 1 – диэтилфталат; 2 – дибутилфталат; 3 – диоуктилфталат; 4 – бутилтетрадецилфталат. Контроль – рост без бактерий, + *Rhizobium* и + *Azotobacter* – инокуляция бактериями *R. leguminosarum* bv. *viceae* and *Azotobacter chroococcum* соответственно. S, % – относительная площадь для пиков фталатов на хроматограмме (ГХ-МС)

medium of the seedling roots occurred due to its active destruction in the cells of this bacterial species. The ability to catabolize N-phenyl-2-naphthylamine with the formation of phthalates was previously determined in *Rhizobium leguminosarum* bv. *viceae* [6].

The composition of the compounds in ethyl acetate extracts from the culture media of bacteria that grew 24 h with 10 μM or 48 h with 100 μM N-phenyl-2-naphthylamine

Состав соединений в этилацетатных экстрактах из культуральных сред с бактериями, росшими 24 ч с 10 μM или 48 ч с 100 μM N-фенил-2-нафтиламином

Compound	RT, min	Ver, %	S, %			
			10 μM		100 μM	
			Rhizobium	*Azotobacter*	*Rhizobium*	*Azotobacter*
Phthalic anhydride	8.0	50.5	0.32	-	0.4	-
Diethyl phthalate	14.1	35.5	-	8.4	1.3	2.6
Butyl octyl phthalate	19.9	11.8	-	3.3		
Dibutyl phthalate	21.6	29.3	3.20	100.0	4.3	0.7
N-phenyl-2-naphthylamine	26.6	47.5	30.20	0.0	100.0	100.0
bis(2-ethylhexyl) phthalate (synonym, dioctyl phthalate)	31.7	71.5	100.00	57.7	0.6	-
bis(7-methyloctyl) phthalate (synonym, diisononyl phthalate)	35.0	65.9	1.20	-	-	-

Note. RT – retention time; Ver.; % – probability; S, % relative peak area. Mean S, % for three experiments are provided ± the standard deviations. R*hizobium – R. leguminosarum bv. viceae; Azotobacter – Azotobacter chroococcum*.

In culture media containing 10 μM N-phenyl-2-naphthylamine, where *Azotobacter chroococcum* was added, the products of its degradation (phthalates) were also found [7]. The difference between the degrading ability of *Azotobacter* compared to *Rhizobium* (with respect to N-phenyl-2-naphthylamine explains the difference in their content in the root growth media under their influence (Tab.). According to Table it is also possible to observe a decrease in the activity of N-phenyl-2-naphthylamine degradation in the studied bacteria at its 100 μM concentration in the medium. This decrease is found when comparing the peak percentages for phthalates and for N-phenyl-2-naphthylamine introduced into the culture media at 10 and 100 μM, as well as when taking into account the different cultivation durations.

Differences in the catabolism activity of aromatic compounds in the compared bacterial species are most likely predetermined at the genetic level. According to the cited literature [23], the inducible synthesis of enzymes involved in the catabolism of these compounds is a characteristic feature of the biodegradation of this type of compounds for *Rhizobium*. The activity during the degradation of N-phenyl-2-naphthylamine in the strain Az dl0 VKM B-2272 D of *Azotobacter chroococcum* used in our experiments is higher than that of rhizobia. It makes it possible to suggest that the synthesis of enzymes, which are necessary for the named compound biodegradation, is expressed constitutively in these bacteria. The constant presence of these enzymes in the cells of the above mentioned strain of *Azotobacter* explains its resistance to deltometrin, one of the indicators which was followed during its selection [24].

The negative effect of N-phenyl-2-naphthylamine on the growth and the viability rhizospheric bacteria including the bacteria *Rhizobium leguminosarum* bv. *viceae* is confirmed by the results of the research [4, 6]. The data presented in Fig. 3 show the different nature of the effect of the compound discussed on the growth of the bacteria *Rhizobium* and *Azotobacter*. When analyzing them, it is necessary to take into account the low content of carbohydrate food source for the bacterial growth in our experiments (see Methodology). This circumstance led to a decrease in their viability on the 2nd day of exposure [7]. The introduction of N-phenyl-2-naphthylamine into the culture medium further decreased the viability indices and they were decreased to a greater extent at 100 μM.

The same oligotrophic nutritional conditions affected the growth indices (CFU) of the control bacteria *Rhizobium* and *Azotobacter* differently. On the 1st day of the growth, the CFU index of *Azotobacter* was 2.5 times higher than that of *Rhizobium*. On the 2nd day of the exposure, this index was almost unchanged in *Azotobacter*, while it was more than 1.2 times lower in *Rhizobium* compared to the 1st day. Relative indices calculated with CFU (% of control,

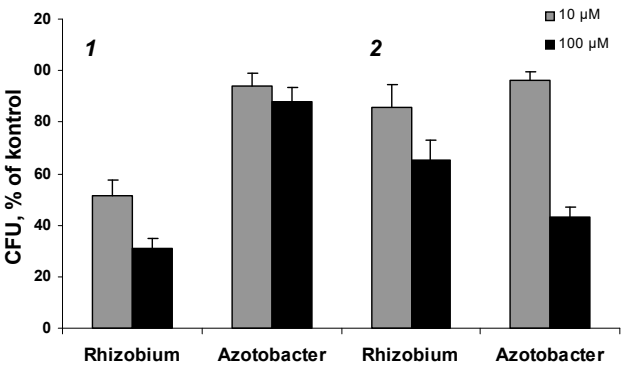

Fig. 3. Percentage of CFU relative to the control for bacteria grown in planktonic cultures without application (control) and with the addition of 10 and 100 μM N-phenyl-2-naphthylamine into the medium for 1(***1***) and 2 (***2***) days. *Rhizobium* and *Azotobacter*, respectively, bacteria *R. leguminosarum* bv. *viceae* and *Azotobacter chroococcum*. CFU – the colonies of forming units

Рис. 3. Процент КОЕ относительно контроля для бактерий, выращенных в планктонной культуре без внесения (контроль) и с внесением 10 и 100 μM N-фенил-2-нафтиламина в среде за 1(***1***) и 2(***2***) суток. *Rhizobium* и *Azotobacter* соответственно бактерии *R. leguminosarum* bv. *viceae* и *Azotobacter chroococcum*. КОЕ – колониеобразующие единицы

see Fig. 3) allow us to trace the occurrence nature of the bacterial growth reaction to the action of 10 and 100 μM N-phenyl-2-naphthylamine during both periods of observation. The effect of N-phenyl-2-naphthylamine on the compared bacterial species differed in the degree of inhibition of their growth at different periods of exposure; it increased at 100 μM. On the 1st day of exposure, *Azotobacter* showed a weak negative reaction to the effect of both concentrations of N-phenyl-2-naphthylamine (differences in the effect of 10 and 100 μM were not significant). A significant suppression of their growth occurred on the 2nd day of the exposure only at 100 μM. The *Rhizobium* bacterial growth was inhibited by N-phenyl-2-naphthylamine to a greater extent on the 1st day; at 100 μM, the growth inhibition increased by about 20% in both periods of the exposure.

The research results make it possible to associate the increased inhibition of the bacterial growth at 100 μM (Fig. 3) with the catabolism inhibition of N phenyl-2-naphthylamine at the same concentration in the bacterial cells (Tab.). Probably, when the concentration of N phenyl-2-naphthylamine is high, its increased accumulation in the bacterial cells leads to the inhibition of their metabolic processes. The physico-chemical properties of the compound, such as high lipophilicity and high antioxidant activity [18, 25], can contribute to this process.

The third group of substances in the field of sight of our research is phthalates; they are also classified as antimicrobial substances. The results of studies that revealed the specific effects of phthalates as in-

hibitors of bacterial growth indicate the role of negative regulators in the plant rhizosphere for phthalates secreted by root cells into the environment [4, 9, 26]. The degree of the negative effect of phthalates was found to be determined not only according to their concentration in the medium, but according to the type of alkyl groups in their molecules linked to *o*-phthalic acid via the ester bond as well. The degree also depends on the species of bacteria experiencing the action of phthalates [19]. The research [9] demonstrated the ambiguous effect of phthalates on the growth of bacteria in planktonic culture and in biofilms. At the same time, many free-living microorganisms are able to use *o*-phthalic acid esters as a source of carbon and energy; that gives them certain selective advantages [27].

According to the data in Fig. 1 and 2, it is possible to trace the changes in the composition of phthalates, which arose under the effect of *Rhizobium* and *Azotobacter* in the growth medium of pea seedling roots, compared to the control. According to the comparison with the control plants, the inoculation with rhizobia led to a slight increase in the amount of dibutyl phthalate and a slight decrease in the percentage of dioctyl phthalate and diethyl phthalate. As affected by azotobacter, along with a decrease in the amount of dibutyl phthalate, the percentage ratio of the other three types of phthalates to this phtalate increased significantly. At the same time, dioctyl phthalate was dominant among the representatives of the group of substances under study. The data [9, 26, 28] demonstrated the antibacterial properties of dioctyl and diethyl phthalates. Taking the data into account, it can be assumed that with the changes in the composition of phthalates, *Azotobacter* grew the potential to increase their competitiveness.

Changes in the phthalate ratios in the growth medium of pea seedling roots as affected by the studied bacterial species can occur due to their possibility to degrade N-phenyl-2-naphthylamine to the phthalate formation and to convert phthalates of one type into other types by changing the length of their alkyl chains linked to o-phthalic acid via the ester bond. The second of the properties was noted by [27] for some representatives of soil bacteria and was confirmed by our studies [6]. The ability of bacteria to convert phthalates of one type into other types can explain the inconsistency of the ratios between the types of phthalates in culture media (Tab.) and in the root growth medium (Fig. 2).

CONCLUSION

Ambiguous trends in the content of the compounds with a negative effect on living organisms, pisatin, N-phenyl-2-naphthylamine, and phthalates, have been determined in the growth medium of pea seedling roots as affected by the bacteria *Rhizobium* and *Azotobacter*. Differences in their content in the root growth medium prove the dissimilar nature of the protective antimicrobial reaction display in the plant under study when interacting with coexistent bacteria of the endophytic and epiphytic types. The increase in the total content of the studied compounds in the growth medium of pea seedling roots 1 day after the inoculation with endosymbiotic bacteria is clear evidence of the increase in their protective reaction. This increase is probably due to the processes characteristic of this period of the legume-rhizobial symbiosis formation: adhesion of rhizobia on the root surface and the beginning of the process of their penetration into the root hairs [29]. Based on the above noticed changes in the total content of compounds with a negative effect, the inhibition of bacterial growth in the root zone of pea seedlings in the case of their interaction with rhizobia is assumed to be quite probable.

A decrease in the amount of the discussed compounds in the root growth medium during the same period of interaction of pea seedlings with *Azotobacter* indicates a weakening of the protective reaction of these plants against the bacteria. It is to be supposed, the conditions for the rhizosphere bacteria existence in the root zone of pea seedlings are more comfortable in this case. The increase in the proportion of antimicrobial phthalates (dioctyl phthalate and diethyl phthalate) found in the root growth medium after the inoculation with *Azotobacter* is likely to increase the competitiveness of this species among rhizosphere bacteria.

To sum up, it is possible to conclude that the concentration of negative substances with antimicrobial activity in plant root growth is not only due to their exudation by the root cells. Rhizosphere bacteria in the root zone also modify their content. In all probability, the level of metabolic activity in bacterial cells during the degradation of aromatic compounds determines the degree of these modifications.

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Contribution of the authors
The authors contributed equally to this article.

Conflict interests
The authors declare no conflict of interests regarding the publication of this article.

The final manuscript has been read and approved by all the co-authors.

Information about the article
The article was submitted 04.03.2022.
Approved after reviewing 14.04.2022.
Accepted for publication 15.09.2022.

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Вклад авторов
Все авторы сделали эквивалентный вклад в подготовку публикации.

Конфликт интересов
Авторы заявляют об отсутствии конфликта интересов.

Все авторы прочитали и одобрили окончательный вариант рукописи.

Информация о статье
Поступила в редакцию 04.03.2022.
Одобрена после рецензирования 14.04.2022.
Принята к публикации 15.09.2022.