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Preface to the Special Issue

Special issue of the «Journal of the Siberian Federal University. Biology» is devoted to regulation of plant redox metabolism at the molecular, biochemical, and physiological levels.

In the modern world, all components of the biota are affected by stressors of different strength and nature, associated with both climatic changes and human impact. Living organisms are forced to adapt to these factors by regulating their vital processes at the genetic, biochemical, and physiological levels. Plants, being the primary producer, determine the functioning of all components in ecosystems, and, therefore, their ability to survive adverse environmental conditions is extremely important for the entire Biosphere. In response to any stressor, an oxidative burst occurs in plant cells, accompanied by the production of reactive oxygen species, which significantly changes the redox metabolism of plants and triggers the corresponding signaling pathways ultimately leading to the adaptation of plants to new conditions.

The articles included in this special issue are based on the presentations of the participants of the III International Symposium «Molecular Aspects of Plant Redox Metabolism» and the School for Young Scientists «The Role of Reactive Oxygen Species in Plant Life», which were held at the Institute of Natural Sciences and Mathematics of the Ural Federal University (Ekaterinburg) in August 2021.

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Предисловие к тематическому выпуску

Специальный выпуск «Журнала СФУ. Биология» посвящен проблемам регуляции редокс-метаболизма растений на молекулярном, биохимическом и физиологическом уровнях.

В современном мире все компоненты биоты испытывают разные по силе и по характеру стрессовые воздействия, связанные как с климатическими изменениями, так и с антропогенным влиянием. К этим факторам живые организмы вынуждены приспосабливаться, осуществляя регуляцию процессов жизнедеятельности на генетическом, биохимическом, физиологическом уровнях. Растения, являясь первичным продуцентом в экосистемах, определяют существование всех компонентов биоценозов, поэтому их способность переживать неблагоприятные условия среды исключительно важна для функционирования всей биосферы. В ответ на любой стрессор в клетках растений развивается окислительный взрыв, сопровождающийся продукцией активных форм кислорода, что существенно меняет редокс-метаболизм растений и запускает соответствующие сигнальные пути, приводящие в итоге к приспособлению растений к новым условиям.

Статьи, вошедшие в этот тематический выпуск, написаны по материалам докладов ученых, принявших участие в III Международном симпозиуме «Молекулярные аспекты редокс-метаболизма растений» и Школе молодых ученых «Роль активных форм кислорода в жизни растений», которые состоялись в августе 2021 года в Институте естественных наук и математики Уральского федерального университета (Екатеринбург).

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The Effect of Habitat Conditions on the Activity of Enzymes and Content of Metabolites of the Ascorbate-Glutathione Cycle in *Plantago media* Leaves

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Abstract. The ascorbate-glutathione cycle (AGC) is a metabolic pathway that detoxifies H_2O_2, which is a reactive oxygen species produced as a waste product in metabolism. The cycle involves the antioxidant metabolites – ascorbate, glutathione, and NADPH, as well as enzymes linking them. We studied the effect of habitat conditions on the activity of enzymes and contents of metabolites of the ascorbate-glutathione cycle in *Plantago media* leaves. The experimental plants grew on the floodplain meadow: on the sparsely vegetated coastal edge (Site 1) and in the grass stand in the central part of the meadow (Site 2). The hoary plantain plants growing in Site 1 received twice more light than the plants in Site 2. The ascorbate and glutathione concentrations in leaves of the well-lit plants were 2–3 times higher than in shaded plants. The maximal levels of these metabolites were observed at midday, when light intensity and air temperature were increased, and relative humidity was decreased. The activity of AGC enzymes was changing similarly to the metabolite contents. As a result, the leaves of hoary plantain plants from the sites with different light levels did not significantly differ in their hydrogen peroxide concentrations. Our data suggest that the environmental conditions and, above all, the light intensity fine-tune the operation of AGC in plant leaves.

Keywords: *Plantago media*, ascorbate-glutathione cycle, enzyme activity, metabolite content, light, environmental conditions.

Acknowledgements. This work was conducted within the framework of the state budget research, development, and technological work «Physiology and Stress Resistance of Photosynthesis of Plants and Poikilohydric Photoautotrophs in the North» (No. AAAA-A17–117033010038–7).

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Влияние условий обитания на активность ферментов и содержание метаболитов аскорбат-глутатионового цикла в листьях *Plantago media*

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Уральского отделения РАН
Российская Федерация, Сыктывкар

Аннотация. Аскорбат-глутатионовый цикл (АГЦ) представляет собой метаболический путь, осуществляющий детоксификацию H_2O_2 в растительных клетках. В цикле участвуют низкомолекулярные антиоксиданты аскорбат и глутатион, НАДФН и ряд ферментов (аскорбатпероксидаза, дегидроаскорбатредуктаза, глутатионредуктаза). Мы исследовали закономерности изменения активности ферментов и содержания метаболитов АГЦ в листьях *Plantago media* (подорожник средний) в зависимости от условий местообитания. Растения произрастали на пойменном лугу: на слабо покрытой растительностью песчаной бровке ближе к реке (участок 1) и в травостое в центре луга (участок 2). Растения участка 1 получали вдвое больше света, чем растения участка 2. Выявили, что листья хорошо освещаемых растений содержали в 2–3 раза больше аскорбата и глутатиона, чем листья затененных растений. Максимальное накопление фонда этих метаболитов наблюдалось в полдень, когда освещенность и температура среды повышались, а относительная влажность воздуха снижалась. Уровень активности ферментов в течение дня изменялся комплементарно содержанию метаболитов. В результате хорошо освещенные и затененные растения не различались существенно по содержанию пероксида водорода в листьях. В целом наши данные показывают, что функционирование метаболического пути, участвующего в поддержании редокс-состояния фотосинтезирующих клеток, зависит от условий среды и, прежде всего, интенсивности света, тонко регулирующего активность ферментов и накопление метаболитов АГЦ.

Ключевые слова: *Plantago media,* аскорбат-глутатионовый цикл, ферменты, метаболиты, свет, условия среды.

Благодарности. Работа выполнена в рамках Госбюджетной научно-исследовательской, опытно-конструкторской и технологической работы «Физиология и стресс-устойчивость фотосинтеза растений и пойкилогидрических фотоавтотрофов в условиях Севера» (№ АААА-А17–117033010038–7).

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Introduction

Plants are constantly adapting to changing environmental conditions and, above all, any light that they are exposed to. The most important source of reactive oxygen species (ROS) in photosynthetic cells is the chloroplast electron transport chain. If too much energy is absorbed relative to the requirements of the photosynthetic process, the generation of ROS is increased. High light, especially when combined with other stressors, leads to the development of photo-oxidative stress and photoinhibition (Foyer, 2018). In the course of evolution, plants have developed different mechanisms to adapt to high photosynthetic photon flux density. They can adjust the angle of their leaves and chloroplast localization in the cells to minimize exposure, decrease chlorophyll content, and dissipate energy via the xanthophyll cycle and other energy dissipation processes (Bukhov et al., 2001; Vogelmann, Gorton, 2014; Ruban, 2015). Plants can prevent excessive ROS accumulation and maintain the cell redox balance by regulating the antioxidant system activity (Halliwell, 2006; Foyer, 2018).

The ascorbate-glutathione cycle (AGC) is an important metabolic pathway that functions in the cytosol, mitochondria, plastids, and peroxisomes (Asada, 2000). The cycle involves ascorbate (Asc), glutathione (GSH), NADPH, and enzymes that bind these metabolites (Fig. 1). Ascorbate peroxidase (APX) uses two ascorbate molecules to reduce hydrogen peroxide (H_2O_2) to water with the concomitant formation of monodehydroascorbate (MDA). MDA is a radical with a short life-time, and can be spontaneously oxidized to dehydroascorbate (DHA) or reduced by the NADPH-dependent enzyme monodehydroascorbate reductase (MDAR) to ascorbate. DHA is reduced rapidly to ascorbate by dehydroascorbate reductase (DHAR), the process involving reducing equivalents – glutathione. NADPH and glutathione reductase (GR) take part in the reduction of oxidized glutathione (GSSG) in the cell (Foyer, Noctor, 2011).

AGC is a key mechanism for regulation of H_2O_2 content in plant cells (Foyer, Noctor, 2011). H_2O_2 is produced mainly during photosynthetic and photorespiratory processes, especially when plants are exposed to strong light. This relatively stable compound is supposed to play a central role in orchestrating plant metabolism, as it can affect expression of many genes and regulate many processes (Neill et al., 2002; Foyer, 2018).

The aim of our study was to estimate the effects of habitat conditions on the activities

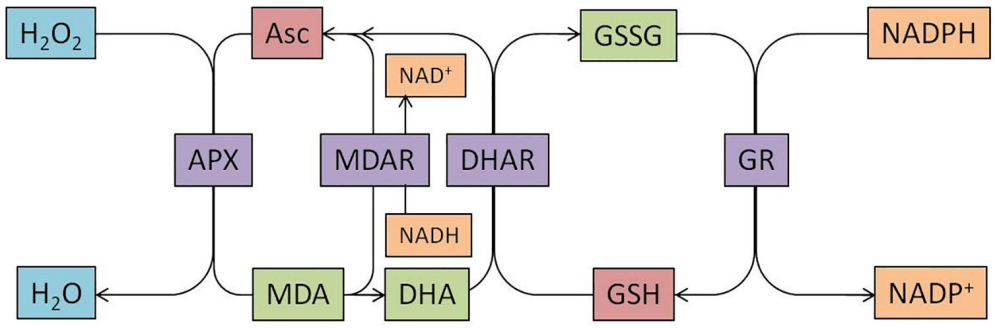

Fig 1. A scheme of the glutathione-ascorbate cycle (Foyer-Halliwell-Asada pathway)

of enzymes and contents of metabolites of the ascorbate-glutathione cycle in *Plantago media* L. leaves. For this, we assessed the daytime changes of ascorbate and glutathione pools and hydrogen peroxide content. We also determined the activities of ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase.

Materials and methods

Plant material

P. media (hoary plantain) is a perennial herbaceous plant of Plantaginaceae family. Experimental plants grew in the Vym river floodplain meadow (62°16'19"N, 50°39'29"E). Plants grew on the sparsely vegetated coastal edge (Site 1) and in the grass stand in the central part of the meadow (Site 2). The soil of the meadow is well-drained sod-layered sandy-sandy loam.

The research was conducted in early July 2018, when plants were in the beginning of the budding stage. We collected fully developed leaves from the middle part of the *P. media* rosette during the day. We placed the samples from 15–25 plants in a dewar with liquid nitrogen, delivered them to the laboratory, and stored at –70 °C prior to the analysis.

Photosynthetic photon flux density (PPFD, μmol m^{-2} s^{-1}), temperature (T, °C) and relative humidity (RH, %) at the level of the hoary plantain leaves were measured with sensors of portable weather station LI-1400 (LI–COR, USA).

Biochemical analyses

Ascorbate peroxidase (EC1.11.1.11) activity was determined according to Nakano and Asada (1981). This method is based on measuring the optical density of the solution during the ascorbate oxidation. Dehydroascorbate reductase (EC1.8.5.1) activity was determined by calculating the rate of dehydroascorbate reduction (Hossain, Asada, 1984). The activity of glutathione reductase (EC1.8.1.7) was determined by NADPH oxidation kinetics in the presence of oxidized glutathione (Foyer, Halliwell, 1976). Soluble protein content was determined according to Bradford (1976). All manipulations with the enzymes were performed at 4 °C.

The ascorbate content was determined based on the reduction of Fe^{3+} to Fe^{2+} (Kampfenkel et al., 1995). The glutathione content was determined according to Queval and Noctor (2007). The H_2O_2 content was determined according to Bellincampi et al. (2000).

Statistical analysis

Statistical analysis of the data was performed using the Statistica 6.1 software («StatSoft Inc.», U.S.A.). Data are shown as the mean ± standard error (SE). Normal distribution was confirmed using the Shapiro–Wilk test. Means were compared using analysis of variance (one-way ANOVA) and Duncan's test. A value of $P < 0.05$ was considered statistically significant.

Results

Microclimatic conditions in plant habitats

The *P. media* species is widely represented in the local flora; it exhibits high morphophysiological plasticity and ability to form phenotypes adapted to various environmental conditions. Hoary plantain plants inhabit floodplain meadows, roadsides, and sparse forests, and this species is a pioneer on shallows and limestone outcrops. We studied plants growing in different locations of the floodplain meadow.

Hoary plantain plants growing in Site 1 received more light and heat than those in Site 2 (Fig. 2). In this site at midday, the average PPFD at the level of the hoary plantain leaves was 600–700 μmol m^{-2} s^{-1}. The air warmed up to 23–25 °C. The plant leaves in Site 2 received 2–3 times less light. The air temperature was also 3–4 °C

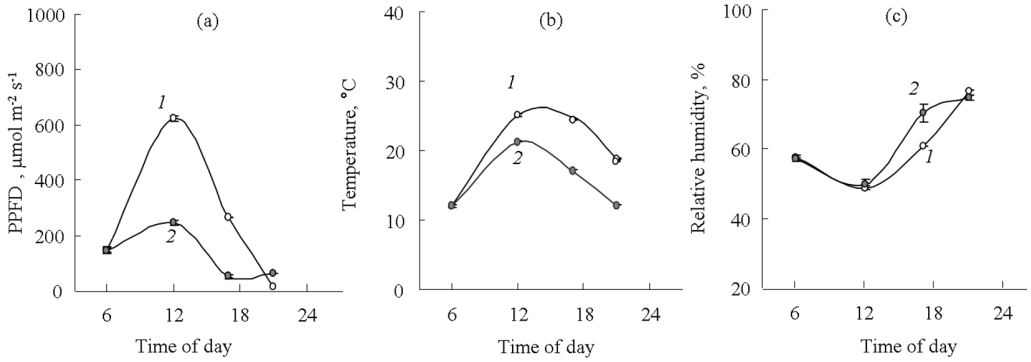

Fig. 2. Diurnal dynamics of photosynthetic photon flux density (a), air temperature (b), and relative humidity (c) in *Plantago media* habitats: (*1*) the sparsely vegetated coastal edge (Site 1) and (*2*) the grass stand in the central part of the meadow (Site 2)

lower than in Site 1. The relative humidity was distinctly higher in the second half of the day in Site 2.

Ascorbate and glutathione contents

Total ascorbate concentration increased by 17 % during the first half of the day in leaves of plants from Site 1 (Table 1). The reduced ascorbate dominated in the total ascorbate pool, amounting to 80 % on average. Ascorbate concentration decreased slightly after midday. Ascorbate concentration in the leaves of plants from Site 2 was lower by a factor of more than two compared to plants in Site 1 and increased slightly after midday.

A 30–40 % increase in reduced glutathione concentration was observed during the first half of the day in hoary plantain leaves from both sites (Table 2). The reduced GSH content decreased 3.5 times in the leaves from Site 1 by the nighttime. Oxidized glutathione constituted 1 % of the total glutathione pool in the daytime. A significant increase in the level of oxidized glutathione concentration was observed in the evening. The daily changes of the glutathione level in plant leaves from Site 2 were similar, but the reduced GSH concentration was lower.

Level of AGC enzyme activities

The ascorbate peroxidase (APX) activity changed markedly during the day in the leaves of plants from Site 1 (Fig. 3a). We observed a 1.3-fold increase in APX activity from early morning to midday and the subsequent decrease.

Table 1. Reduced and oxidized ascorbate concentrations in the leaves of the hoary plantain plants, μmol g^{-1} dry weight

Time of day	Site 1			Site 2		
	Reduced ascorbate	Oxidized ascorbate	Total content	Reduced ascorbate	Oxidized ascorbate	Total content
6	$29.1 \pm 0.6^{a*}$	5.5 ± 0.6^{a}	$34.0 \pm 0.6^{a*}$	12.0 ± 0.3^{a}	5.0 ± 0.6^{a}	17.0 ± 0.5^{a}
13	$34.3 \pm 1.5^{b*}$	$8.4 \pm 0.8^{b*}$	$41.4 \pm 2.0^{b*}$	12.4 ± 0.2^{a}	4.8 ± 0.4^{a}	17.7 ± 0.2^{a}
22	$30.8 \pm 0.6^{a*}$	$8.3 \pm 1.2^{b*}$	$39.9 \pm 1.3^{b*}$	13.9 ± 0.6^{b}	5.4 ± 0.7^{a}	19.3 ± 1.0^{b}

Asterisks indicate significance of differences of Site 1 from Site 2. Different superscript letters denote statistically significant changes throughout the day ($P < 0.05$; Dunkan-test).

Table 2. Reduced and oxidized glutathione concentrations in the leaves of hoary plantain plants, μmol GSH g^{-1} dry weight

Time of day	Site 1			Site 2		
	Reduced glutathione	Oxidized glutathione	Total content	Reduced glutathione	Oxidized glutathione	Total content
6	$5.6 \pm 0.9^{ab*}$	0.05 ± 0.01^{a}	$5.7 \pm 0.5^{a*}$	2.2 ± 0.5^{a}	0.08 ± 0.01^{a}	2.3 ± 0.3^{a}
13	$9.3 \pm 1.5^{a*}$	$0.09 \pm 0.01^{a*}$	$9.4 \pm 0.4^{b*}$	3.2 ± 1.3^{b}	0.13 ± 0.01^{a}	3.3 ± 0.1^{b}
22	2.6 ± 0.1^{b}	$0.45 \pm 0.01^{b*}$	2.9 ± 0.5^{c}	1.9 ± 0.2^{ab}	0.51 ± 0.01^{b}	2.4 ± 0.1^{a}

Asterisks indicate significance of differences of Site 1 from Site 2. Different superscript letters denote statistically significant changes throughout the day ($P < 0.05$; Dunkan-test)

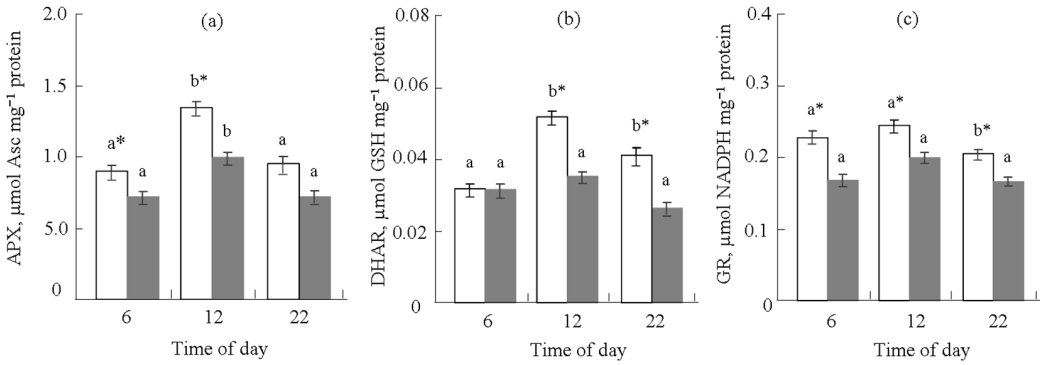

Fig. 3. Ascorbate peroxidase (a), dehydroascorbate reductase (b), and glutathione reductase activities (c) in the leaves of hoary plantain plants from Site 1 (light columns) and from Site 2 (dark columns). Asterisks indicate significance of differences of Site 1 from Site 2. Different superscript letters denote statistically significant changes throughout the day ($P < 0.05$; Dunkan-test)

The daytime course of the APX activity in the leaves of hoary plantain from Site 2 was similar, but the level of the APX activity was lower by 20 %.

The level of dehydroascorbate reductase (DHAR) activity increased from early morning to midday by 40 % in the leaves of plants from Site 1 (Fig. 3b). No significant daily changes in the activity of this enzyme were observed in plants from Site 2. The level of the DHAR activity in leaves of hoary plantain from Site 2 was lower compared to plants from Site 1.

The level of glutathione reductase (GR) activity did not change during the day in the leaves of plants from Site 2, while in the plants from Site 1 it decreased by 30 % by the nighttime (Fig. 3c). During the daytime, the level of the GR activity in leaves of plants from Site 1 was slightly higher than in the leaves from Site 2.

Hydrogen peroxide content

The habitat conditions did not have a significant effect on the H_2O_2 concentration in the leaves of hoary plantain (Fig. 4). In both sites, the concentration of H_2O_2 in the daytime was 30–40 % higher than in the nighttime, but plant leaves from Site 2 contained slightly less hydrogen peroxide compared to plants from Site 1.

Discussion

Peroxide is produced mainly during photosynthetic and photorespiratory processes, especially when plants are exposed to strong light. From our experiments it is obvious that

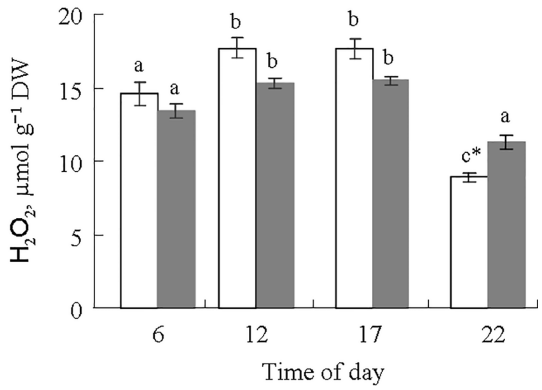

Fig. 4. Hydrogen peroxide concentration in the leaves of hoary plantain plants from Site 1 (light columns) and from Site 2 (dark columns). Asterisks indicate significance of differences of Site 1 from Site 2. Different superscript letters denote statistically significant changes throughout the day ($P < 0.05$; Dunkan-test)

the leaves of hoary plantain plants from the sites with high and low light did not differ significantly in hydrogen peroxide concentration (Fig. 4). Presumably, this may be the result of ascorbate-glutathione cycle activity, which is an important metabolic pathway for detoxification of hydrogen peroxide (Asada, 2000). The ascorbate peroxidase reaction is the first in AGC. This enzyme uses ascorbate to reduce H_2O_2 to water. Our data show that leaves of the plants receiving more light contained twice as much ascorbate compared to the shaded plants from Site 2.

Ascorbate is a low-molecular-weight antioxidant. The antioxidant properties of ascorbate are related to the one-electron cyclic transformation between the reduced and oxidized forms of this metabolite (Foyer, Noctor, 2011). Ascorbate reacts directly with H_2O_2, NO•, and $O_2^{\bullet-}$ and can break the chain reactions of oxidation of organic molecules. Ascorbate takes part in the reduction of other low-molecular-weight antioxidants (α-tocopherol, glutathione, phenolic compounds) (Smirnoff, 2000; Radyukina et al., 2019). Ascorbate is a substrate for ascorbate peroxidase in cytosol and chloroplasts (Foyer, Noctor, 2011). Ascorbate plays an important role in photoprotection as a cofactor of violoxanthin de-epoxidase in the xanthophyll cycle (Conklin, 2001), and as key regulator of anthocyanin synthesis (Plumb et al., 2018). Previously, we showed that the hoary plantain plants from open habitats had the higher level of xanthophyll cycle pigment conversion compared to shaded plants (Golovko et al., 2012).

The daytime changes in the ascorbate content were detected in hoary plant leaves. Our results are consistent with the data reported by other authors (see review of Foyer, Noctor, 2011). The reduced form of ascorbate was dominant in the total ascorbate pool of the leaves from both sites, indicating the effective operation of the regeneration systems of this metabolite. As is well known, in addition to the *de novo* synthesis, the oxidized form of ascorbate is reduced in glutathione-dependent reaction with dehydroascorbate reductase (DHAR). According to our data, DHAR activity was higher in the leaves of plants from Site 1, where the lighting was twice higher. This indicates the importance of maintaining a high level of the reduced ascorbate in the photosynthetic cells under the high light condition. The analysis of the results revealed the presence of a statistically significant correlation ($r = 0.98$) between the ascorbate concentration and the DHAR activity in the leaves of *P. media* from Site 1.

Glutathione is an electron donor for DHAR. Glutathione is able to reduce the sulfhydryl groups of proteins after their oxidation by H_2O_2. Glutathione is an important participant in redox signaling (Foyer, Noctor, 2011). The results of our experiments showed that the leaves of *P. media* from Site 1 contained significantly more glutathione than the leaves of shaded plants from Site 2. These data can indicate the importance of this metabolite for maintaining redox homeostasis of the photosynthetically active cells. The accumulation of glutathione in the leaves of plants from Site 1 may be due to their active photorespiration. As is well known, glycine formed during the photorespiration is a substrate to the GSH biosynthesis (Rakhmankulova, 2018). According to our data, the reduced glutathione constituted 80–90 % of the total glutathione pool in *P. media* leaves.

Conclusion

We found that the content of metabolites and activity of the enzymes of the ascorbate-glutathione cycle in the shaded *P. media* plants were significantly lower compared to the plants from the high light habitat. Environmental conditions and, above all, light intensity fine-tune the operation the AGC activity in plant leaves. The obtained results suggest that the effective functioning of the ascorbate-glutathione cycle is a characteristic feature of the metabolism of plant leaves and an important component of the adaptive response, which contributes to the maintenance of the redox balance in the photosynthetic cells.

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Foliar Content of Phenolic Compounds in *Platanthera bifolia* from Natural and Transformed Ecosystems at Different Stages of Orchid Development

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Abstract. The representatives of the family Orchidaceae Juss. are often used as a source of natural antioxidants, including phenolic compounds, which play an important role in plant resistance under stressful conditions. This study investigates the content of lipid peroxidation products and soluble phenolic compounds in flowering plants of *Platanthera bifolia* (L.) Rich. growing in natural (forest park) and transformed (fly ash dumps of Thermal Power Stations) ecosystems of the Middle Urals, Russia, as well as the content of flavonoids at different stages of orchid development. Research has shown that in disturbed habitats, *P. bifolia* is capable of forming abundant populations containing a significant portion of the flowering plants. Additionally, flowering orchids from fly ash dumps contained an average 20 % more lipid peroxidation products, which indicated a shift in the redox balance towards oxidative processes. An increase by 2.4 times on average in the content of phenolic compounds, particularly flavonoids, was observed at all developmental stages of the plants growing in the transformed ecosystems. Regardless of the growing conditions, the non-flowering mature individuals were characterized by a minimum content of flavonoids, probably due to pre-generative metabolic restructuring. Yet, in the period of orchid blooming, the flavonoid content in their leaves increased again in all study sites. At the same time, the flavonoid proportion of the total soluble phenolic compounds was 42 % in the natural habitat, increasing to 66 % on average in the transformed ecosystems. Thus, flavonoids are involved

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in the protective adaptive responses of *P. bifolia*, not only ensuring the survival of this orchid but also contributing to the implementation of its ontogenetic program.

Keywords: Orchidáceae, fly ash substrates, oxidative stress, redox balance, antioxidants, flavonoids, age structure of population.

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Содержание фенольных соединений в листьях *Platanthera bifolia* из естественной и трансформированных экосистем на разных стадиях развития орхидеи

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Аннотация. Представители семейства Orchidaceae Juss. нередко являются источником природных антиоксидантов, к числу которых относятся фенольные соединения, играющие важную роль в формировании устойчивости растений к стрессовым факторам. Изучено содержание продуктов перекисного окисления липидов (ПОЛ) и растворимых фенольных соединений у генеративных особей *Platanthera bifolia* (L.) Rich., произрастающих в естественной (лесопарк) и трансформированных (золоотвалы ГРЭС) экосистемах Среднего Урала, а также содержание флавоноидов в листьях орхидеи на разных стадиях ее развития. Обнаружено, что в нарушенных местообитаниях *P. bifolia* способна формировать ценопопуляции с высокой численностью и значительным вкладом в возрастной спектр генеративных особей. При этом цветущие орхидеи с золоотвалов содержали в среднем на 20 % больше продуктов ПОЛ, что свидетельствует о сдвиге

редокс-баланса в сторону окислительных процессов. Кроме того, у растений, произрастающих в трансформированных экосистемах, наблюдалось увеличение содержания фенольных соединений, в частности флавоноидов (в среднем в 2,4 раза), на всех изученных стадиях. Независимо от условий произрастания виргинильные особи характеризовались минимальным содержанием флавоноидов, вероятно, из-за метаболических перестроек в период закладки генеративных органов. В период цветения количество флавоноидов в листьях орхидеи снова увеличивалось на всех участках. При этом доля флавоноидов от общего содержания фенольных соединений возрастала от 42 % в естественном фитоценозе до 66 % в среднем в трансформированных экосистемах. Сделано заключение, что флавоноиды участвуют в защитно-приспособительных реакциях *P. bifolia*, не только обеспечивая выживание этой орхидеи, но и способствуя реализации ее онтогенетической программы.

Ключевые слова: Orchidáceae, зольные субстраты, окислительный стресс, редокс-баланс, антиоксиданты, флавоноиды, возрастная структура популяции.

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Introduction

In recent decades, the search for promising sources of plant raw materials for preparing effective formulations with a high content of biologically active compounds has become increasingly important (Gutierrez, 2010). Phenolic compounds are unique substances of secondary metabolism with various properties and play an essential role in plants. This is a very diverse group of compounds, which differ in their chemical structure, varying from simple molecules such as phenolic acids to highly polymerized compounds such as condensed tannins. It is well known that the categorization of a compound is based on the number of constituent carbon atoms in combination with the structure of the main phenolic skeleton (Michalak, 2006; Fraga et al., 2009). Moreover, the diversity of the chemical structures of phenolic compounds determines the wide range of properties and functions not only in the cells, but also in the entire plant organisms. For example, lignin plays an essential role in the structural organization of plant cell walls (Landolino, Cook, 2009). Tannins present in vacuoles take part in plant defense against pathogens. Other phenolic compounds, such as phenylpropanoids, protect cell structures against the chain reactions of free radicals due to excessive light; thus, they act as

antioxidants (Babenko et al., 2019). Simple phenols are also widely distributed phenolic compounds, which play an important biological role. Some of them (salicylic acid) act as signaling molecules; others (for example, *n*-hydroxybenzoic acid) are precursors of compounds such as ubiquinones and plastoquinones (Marchiosi et al., 2020).

Flavonoids are the most abundant group of plant polyphenols. These substances play a significant role in the processes of cell signaling, can serve as messengers of chemical signals, and participate in the processes of plant reproduction (Takahama, Oniki, 2000; Pourcel et al., 2007). They are also involved in protecting plants against various adverse environmental factors. One of the most notable functions of these secondary metabolites is plant protection against oxidative stress due to their pronounced antioxidant activity (Takahama, Oniki, 2000; Pourcel et al., 2007; Tarakhovsky et al., 2013).

Different species of the Orchidaceae family are known as the sources of natural antioxidants (Gutierrez, 2010). Many of them have long been used in traditional medicine. However, the chemical composition of orchids as potential sources of biologically active substances has been insufficiently studied. In addition, there is practically no information on the protective antioxidant responses of orchids growing in industrially disturbed territories.

Platanthera bifolia (L.) Rich., commonly known as the lesser butterfly-orchid, is one of the orchid plants with pronounced medicinal properties (Shreter, 1975). It is a protected orchid species listed as «rare» (category III) in many regional Red Data Books of Russia. In the Red Book of the Sverdlovsk Region, though, this species is in category V as «rehabilitating» (Red Book…, 2018). At the same time, in recent years, populations of *P. bifolia* have been found in industrially disturbed areas of the Middle Urals and other regions (Filimonova et al., 2014; Mishagina, 2018; Romanova, Mongush, 2019). Our study focuses on the numerous population of *P. bifolia* found in forest communities formed on the fly ash dumps of Thermal Power Stations (TPS) of the Sverdlovsk Region (Filimonova et al., 2014).

The aim of the study was 1) to estimate the content of lipid peroxidation products and the total soluble phenolic compounds in leaves of *P. bifolia* from natural (forest park) and transformed (fly ash dumps of the TPS) ecosystems of the Middle Urals, Russia; and 2) to compare the foliar flavonoid content at different stages of orchid development.

Materials and methods

P. bifolia is an herbaceous perennial orchid plant with a thickened fusiform stem-root tuberoid; it is a mesophyte, a European–West Asian boreal-nemoral species. It exhibits broad ecological tolerance, but is not strictly confined to a certain plant community (Vakhrameeva et al., 2008; Mishagina, 2018).

In this work, local populations of *P. bifolia* from three different forest communities of the Sverdlovsk Region, Russia (subzone of southern taiga), were studied. Site 1 was located in the natural plant community of the Southwestern Forest Park in the city of Ekaterinburg (56º46'25"N; 60º32'32"E). Site 2 was located in the transformed territory with a forest community (age 45–50 years) shaped like a strip along the inner dam of the fly ash dump of the Sredneural'skaya Thermal Power Station near the town of Sredneural'sk (SUTPS, 57°0'37"N; 60°27'58"E). Site 3 was represented by a forest community (age 35–40 years) naturally colonizing fly ash dump of the Verkhnetagil'skaya Thermal Power Station (VTTPS), the town of Verkhniy Tagil (57°20'46"N; 59°56'45"E) (Fig. 1).

Geobotanical characteristics such as crown density, total shrub projective cover, total

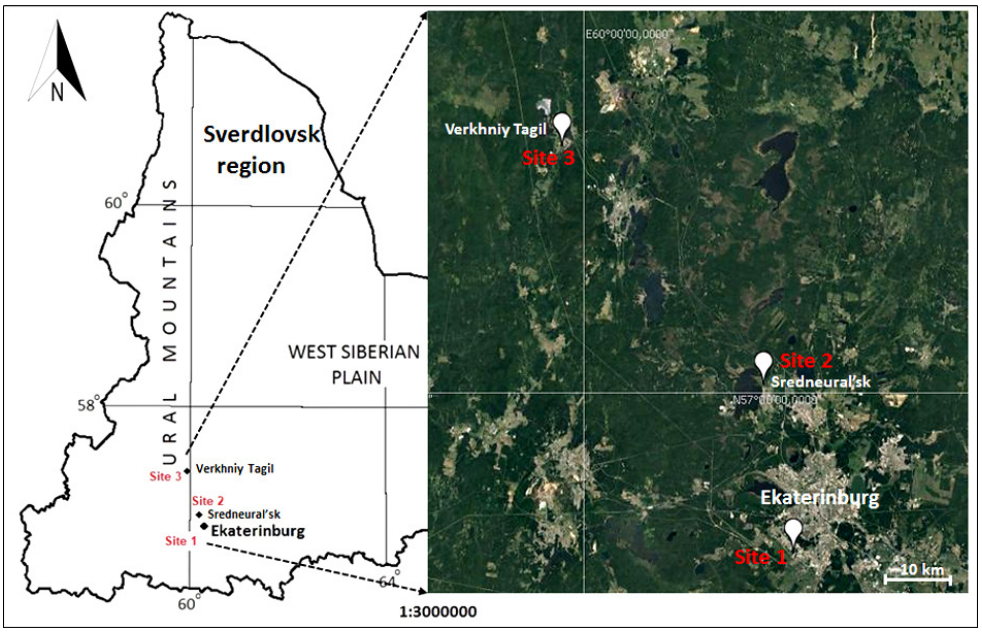

Fig. 1. The schematic map of the Sverdlovsk Region and sampling points of *P. bifolia*

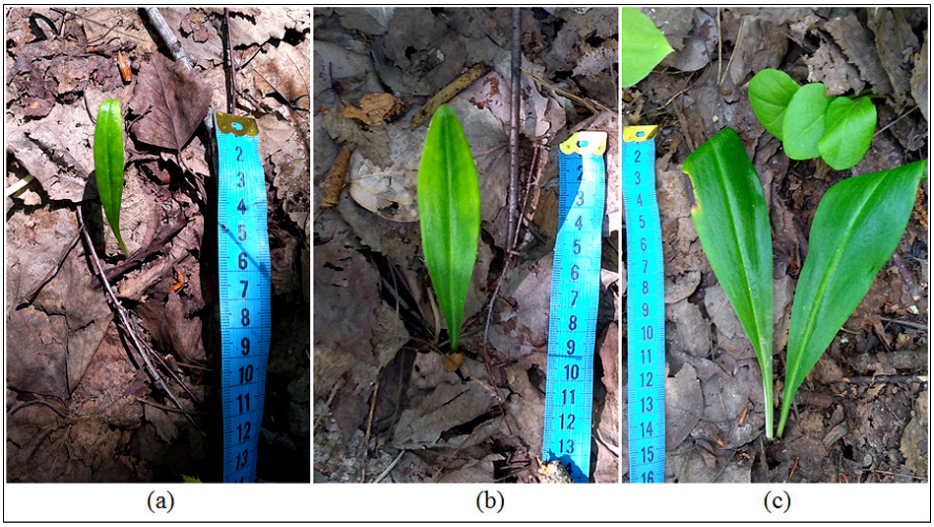

Fig. 2. Age groups of *P. bifolia* individuals: (a) juvenile, (b) immature, and (c) non-flowering mature

herbaceous projective cover, and total projective cover of the moss-lichen layer were studied using standard methods.

In each *P. bifolia* population, the juvenile, immature, non-flowering mature (Fig. 2), and flowering (Fig. 3) developmental stages of individuals were studied.

A juvenile plant had at least one narrow-lanceolate leaf up to 5–6 cm long and about 1 cm wide, with 2–4 veins (Fig. 2a). An immature plant was represented by a small shoot with one lanceolate-elliptical leaf up to 8–10 cm long and up to 2.5 cm wide, with 6–8 veins (Fig. 2b). A non-flowering mature plant had two almost opposite

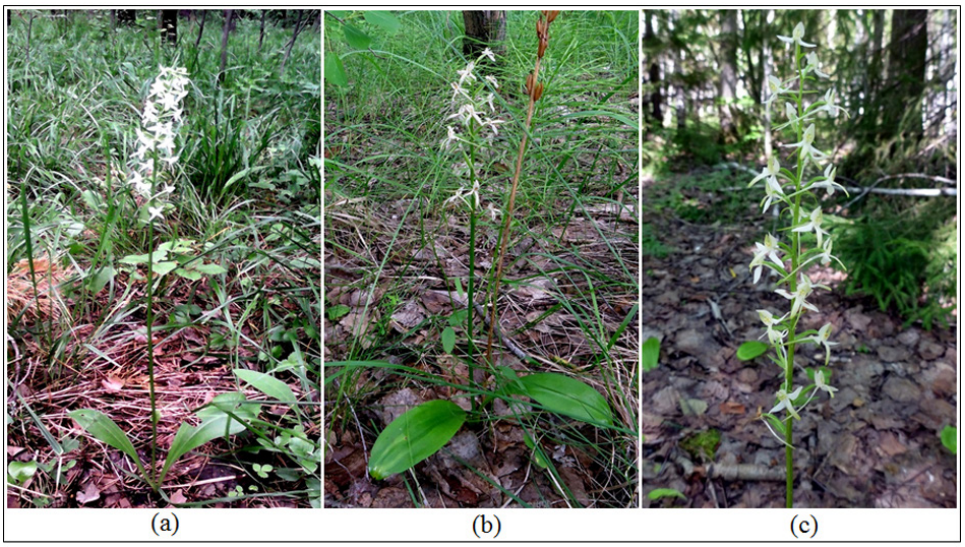

Fig. 3. The individuals of *P. bifolia* at the flowering stage: (a) in natural forest community, (b) on fly ash dump of SUTPS, and (c) on fly ash dump of VTTPS

leaves (rarely one leaf) – elliptical or oblong-elliptical leaves up to 16 cm long and 3.5–4.0 cm wide, with 8–10 veins (Fig. 2c). A flowering plant was represented by a generative shoot with two (less often one) elliptical or oblong-elliptical leaves, up to 20 cm long and 4.5–6.0 cm wide, with 10–12 veins, and inflorescence with 12–20 (occasionally up to 30–35) flowers (Fig. 3).

Leaf samples were collected in mid-July, during the blooming period of orchids. For determination of lipid peroxidation products (malondialdehyde, MDA) and soluble phenolic compounds, the leaves of flowering *P. bifolia* plants were collected from four plants from each study site. For the measurement of flavonoids, the leaves were collected at different stages of development.

Lipid peroxidation was estimated according to Heath and Packer (1968) by measuring the total thiobarbituric acid reactive products at 532 and 600 nm and calculated as MDA content.

The soluble phenolic compounds were determined using the Folin-Ciocalteu assay (Singleton et al., 1999). Briefly, 0.1 mL of crude 96 %-ethanol extract was mixed thoroughly with 0.5 mL of the Folin-Ciocalteu reagent for 3 min, followed by the addition of 0.4 mL of 7.5 % (*w/v*) sodium carbonate. The mixture was allowed to stand for 60 min in the dark, and absorbance was measured at 760 nm. Gallic acid was used as a reference standard.

For determination of flavonoids, 0.5 g of fresh leaf sample was crushed, extracted with 96 % ethanol solution containing 1 % Triton X-100 (*v/v*), and finally filtered through a paper filter. The flavonoid content was measured spectrophotometrically at 420 nm after reaction with citroborat reagent (a mixture of 20 % citric acid and 5 % boric acid at a ratio of 1:1) as described by Rogozhin (2006). A standard curve was plotted using rutin as a standard.

All parameters were measured using fresh plant material in four biological and three analytical replicates and then calculated as per g of dry weight (DW). In order to estimate the DW, the specific amount of fresh leaves was dried at 75 °C for 24 h. The significant difference between the study sites was determined by one-way

Table 1. General geobotanical characteristics of the study sites

Characteristics	Site 1	Site 2	Site 3
Crown density	0.6–0.7	0.4–0.5	0.5–0.6
Total shrub projective cover, %	15–20	15–35	20–30
Total herbaceous projective cover, %	65–80	40–60	10–25
Total projective cover of the moss-lichen layer, %	10–15	5–10	5–8
Number of orchid individuals per 1 m^2	0.4	1.3	2.7

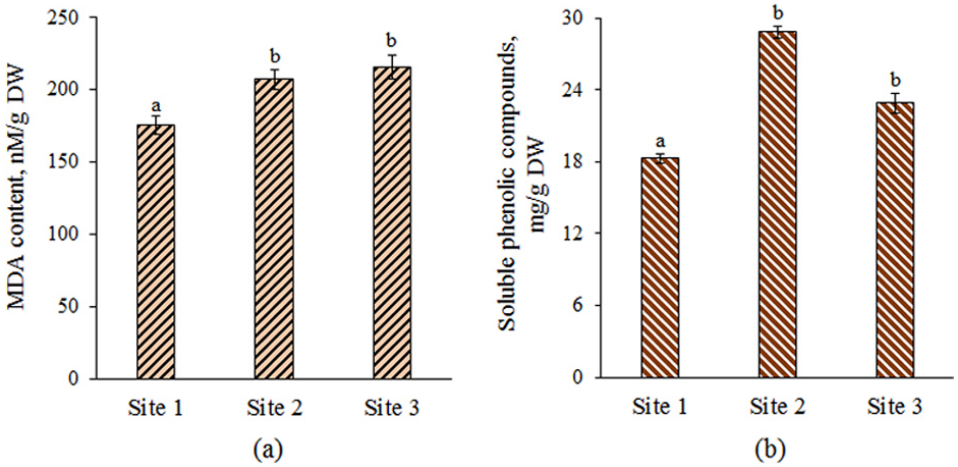

Fig. 4. The foliar content of (a) malondialdehyde (MDA) and (b) soluble phenolic compounds in flowering individuals of *P. bifolia* from natural (Site 1) and transformed (Sites 2 and 3) ecosystems. Data represent means ± SE (n = 12). Different letters indicate significant differences between the study sites at $p < 0.05$

ANOVA followed by Tukey's test ($p < 0.05$). The range values of some geobotanical characteristics of the study sites are listed in Table 1. Data shown in Fig. 4 and 5 are the means ± standard errors (SE).

Results and discussion

The natural recreation zone located far away from the industrial city activities was taken as a control habitat (Site 1) and represented by a 115–130-year-old forest. The soil of this site was sod-medium podzolic (Filimonova et al., 2020). Among the woody species, *Pinus sylvestris* L. dominated, and *Betula pendula* Roth and *Populus tremula* L. were co-dominants. The height of the first layer trees was 20–30 m and the height of the second layer trees was 10–15 m, with a tree crown density of 0.6–0.7 (Table 1). *Salix caprea* L. and *Sorbus aucuparia* L. were found in the underwood. Total shrub projective cover was 15–20 % and contained the dominant *Rubus idaeus* L. and *Rosa acicularis* Lindl. The total herbaceous projective cover was 65–80 %, dominated by *Vaccinium myrtilis* L., *V. vitis-idaea* L., *Aegopodium podagraria* L., *Calamagrostis arundinaceae* (L.) Roth, *Orthilia secunda* (L.) House, *Vicia sylvatica* L., *Maianthemum bifolium* (L.) FW Schmidt, etc. The moss-lichen layer was not well developed whereas leaf litter was poorly developed or absent. *P. bifolia* plants were found both as single individuals and in scattered groups on an area of 400 m^2. Orchid individuals grew at

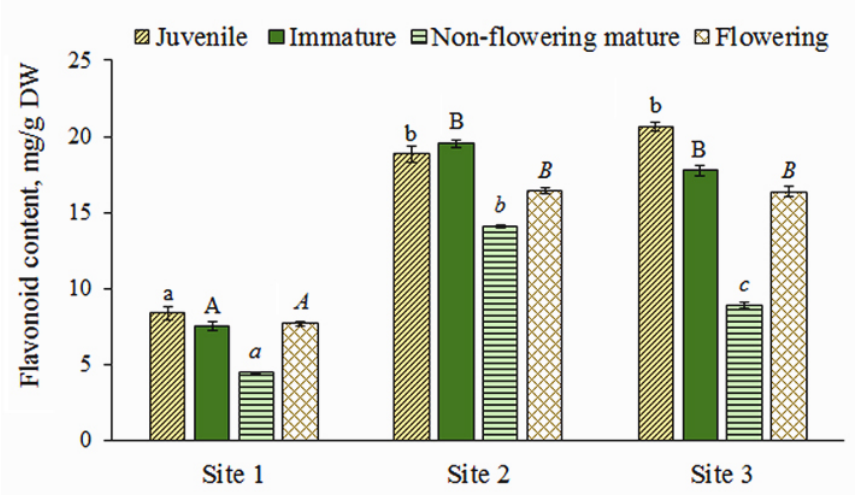

Fig. 5. The foliar flavonoid content at different developmental stages of *P. bifolia* from natural (Site 1) and transformed (Sites 2 and 3) ecosystems. Data represent means ± SE (n = 12). Different small and capital letters indicate significant differences between the study sites at $p < 0.05$

the edge, surrounded by woody plants, in shaded conditions. The total number of plants in control population (P1) was 163 (Table 1).

Site 2 was represented by sparse growth of trees, which naturally colonized the southeast part of the SUTPS fly ash dump. *P. sylvestris*, *B. pendula*, and *P. tremula* were dominant tree species in this forest. The tree crown density varied from 0.4 to 0.5 with the height of the trees between 18 and 20 m. The second layer was dominated by *P. tremula*, *S. caprea*, *S. aucuparia*, *Salix myrsinifolia* Salisb., *Padus avium* L., *Viburnum opulus* L., *R. acicularis* and *Caragana arborescens* Lam. Total shrub projective cover varied from 15 to 35 % whereas the total herbaceous projective cover was 40–60 % (in some places reaching 80–100 %) and was represented by *O. secunda*, *Pyrola rotundifolia* L., *Hieracium umbellatum* L., *P. bifolia*, *Trifolium pratense* L., *Amoria repens* (L.) C. Presl, *Vicia cracca* L., *Poa angustifolia* L., *P. pratensis* L., *Festuca rubra* L., and *Equisetum pratense* L. No significant development of moss-lichen layer was observed. The orchid population (P2) grew on sunny open glades (400 m^2 area) and contained about 518 individuals (Table 1).

Site 3 showed a rather dense orchid population (P3) in the forest naturally colonizing the VTTPS fly ash dump area (150 m^2). The forest was represented by small-leaved trees dominated by *B. pendula*, *P. tremula*, and *P. sylvestris* with the total crown density between 0.5–0.6. The undergrowth included *B. pubescens*, *P. obovata*, and *Larix sibirica* Ledeb. Total shrub projective cover was 20–30 %, while total herbaceous projective cover was 10–25 % and was represented by *Calamagrostis epigeios* (L.) Roth, *P. pratensis*, *A. repens*, *F. rubra*, *Deschampsia cespitosa* (L.) Beauv, *Fragaria vesca* L., *Chimaphila umbellata* (L.) W. Barton, *Pyrola rotundifolia* L., *O. secunda*, *Pyrola chlorantha* Sw., *Chimaphila umbellata* (L.) W. Barton, and *P. bifolia*. Mosses grew only near tree trunks. The population (P3) of *P. bifolia* grew in a sparse forest under diffused lighting conditions, with a total population of 411 individuals (Table 1).

Our study showed that all local orchid populations were normal, incomplete, and capable of self-maintenance (Table 2). *P. bifolia*

Table 2. Structure of *P. bifolia* populations from natural forest community (P1), fly ash dump of SUTPS (P2), and fly ash dump of VTTPS (P3)

Number of individuals at different stages	P1	P2	P3
Juvenile	21	77	62
Immature	33	73	138
Non-flowering mature	49	171	118
Flowering	60	197	93

plants grew in small groups, with the flowering individuals in the center (Fig. 3) surrounded by juvenile, immature, and non-flowering mature plants (Fig. 2). A similar distribution of *P. bifolia* was noted by other authors (Stetsuk, 2010). In the orchid populations in Site 1 (P1), the flowering individuals constituted 37 %, while the juveniles, immature, and non-flowering mature individuals made up 13, 20, and 30 %, respectively. The portion of flowering *P. bifolia* plants in transformed habitats was smaller (23 %) only on Site 3 (P3). A more significant contribution to this population was made by immature and non-flowering mature individuals (62 %).

As is known, the fly ash substrates have unfavorable composition, pH, and other physicochemical properties, a reduced number of microorganisms and fungi, insufficient supply of nutrients (especially nitrogen), and elevated concentrations of some heavy metal(oid)s such as As, Cd, Cr, Hg, Pb, etc. (Chibrik et al., 2016; Gajic et al., 2018). At the same time, the size of *P. bifolia* populations in the fly ash dumps was significantly greater than in the forest park (Table 1). Plants growing on such substrates often experience significant stress, which leads to increased oxidation processes due to overproduction of reactive oxygen species (ROS) in their cells. This is evidenced by the high level of lipid peroxidation, in particular the content of malondialdehyde – a product of membrane lipid degradation.

The level of lipid peroxidation in orchid leaves growing on fly ash substrates was found to be slightly higher (by 20 % on average, Fig. 4a). This indicates the negative effect of the conditions in these industrially disturbed habitats. According to Gajic et al. (2016), in the leaves of *Festuca rubra* grown on fly ash deposits, the content of MDA was high probably as a result of toxic concentrations of As and B and the low content of Cu, Zn, and Mn (Gajic et al., 2016).

Adaptive plant responses to industrially induced stress include changes in metabolism due to the activation of biosynthesis of non-enzymatic antioxidants such as natural phenols. They bind ions well and form complexes with them, preventing the formation of ROS. They are also able to give up a hydrogen atom from the OH-group of the aromatic ring to eliminate free radicals oxidizing lipids and other biomolecules (Michalak, 2006). Therefore, it is interesting to compare the total contents of phenolic compounds in plants from natural and disturbed habitats.

The content of soluble phenolic compounds in the leaves of flowering plants growing in fly ash dumps (Sites 2 and 3) was on average 1.4 times higher than in the natural plant community (Fig. 4b). Results of the present study were in good agreement with the data of other authors (Gajic et al., 2013, 2018), who reported a significant increase in the phenolic compound contents of the leaves and roots of different plant species growing on fly ash deposits.

As noted previously, such phenolic compounds as flavonoids perform a number of functions in the life cycle of plants. This fact is associated with the ability of flavonoids to inactivate ROS, thus preventing the development of oxidative stress and increasing the tolerance of plants to stressful conditions. Their protective role, as a rule, is expressed as an increase in the biosynthesis of these compounds in response to unfavorable factors (Takahama, Oniki, 2000; Khramova et al., 2006; Pourcel et al., 2007).

The results of determining the foliar flavonoid content at different stages of *P. bifolia* development in the natural forest community and disturbed habitats are shown in Fig. 5.

The ontogenetic state can be considered as a key point in the plant development. At the same time, there are very few works addressing the features of physiological processes in different age groups of plants under the anthropogenic stress (Polovnikova, Voskresenskaya, 2008). Therefore, the assessment of physiological parameters at different stages of plant development is of particular relevance.

In the present study, the foliar flavonoid content of *P. bifolia* plants from the fly ash dumps (Sites 2 and 3) was on an average 2.4 times higher compared to the plants from the natural habitat (Site 1). Flavonoids constituted 42 % of the total soluble phenolic compounds in orchid plants from the forest park but 66 % in the plants from the fly ash dumps.

The increased content of flavonoids in the plants from disturbed areas indicates a change in the metabolic processes and phytochemical composition of plants, which are forced to adapt to their environment. Other authors also noted an increase in the flavonoid content in the plants affected by industrial (Nemereshina, Gusev, 2004) and radioactive (Khramova et al., 2006) contamination. For example, under increasing anthropogenic stress, the content of flavonoids and other phenolic compounds in plants was negatively correlated with the soil concentrations of macronutrients (calcium, magnesium, manganese) but positively correlated with concentrations of several trace elements (aluminum and copper) (Artemkina, 2010).

Another possible reason for the increase in the flavonoid content of the orchids from disturbed habitats may be a higher level of light exposure in the fly ash dumps due to the lower total herbaceous projective cover (Table 1). As is known, light induces activation of a number of enzymes that take part in the biosynthesis of phenolic compounds in plants (Zaprometov, 1996).

The present study demonstrated that changes in the flavonoid content in *P. bifolia* leaves occurred in all sites and depended on the age of the plants (Fig. 5). The lowest level of accumulation of these antioxidants was noted in the non-flowering mature stage. An obvious explanation is the depletion of the flavonoid pool due to an imbalance between the processes of flavonoid synthesis and consumption during the period of generative organ formation, when the requirements of plants for active biomolecules are the highest.

Conclusions

In the transformed habitats, the orchid *P. bifolia* is capable of forming abundant populations containing a significant portion of the flowering plants. Regardless of the growing conditions, the non-flowering mature individuals were characterized by a minimal content of flavonoids, probably due to pre-generative metabolic restructuring.

Increased biosynthesis of soluble phenolic compounds, particularly flavonoids, in response to oxidative imbalance in leaves of the *P. bifolia* growing on fly ash substrate was observed at all stages of development studied here. This indicates

high adaptive potential of the orchid *P. bifolia* to endure unfavorable conditions prevailing on fly ash deposits. Natural phenolic compounds are the important antioxidants in plants. A significant increase in their content in *P. bifolia* from fly ash dumps points to the conclusion that they take part in protective adaptive responses, which not only ensure the survival of *P. bifolia* but also contribute to the implementation of its ontogenetic program.

The possibilities of using these orchids in medicine as potential sources of antioxidants and other biologically active substances are limited because of the lack of raw material and the special protected status of orchids. Therefore, the introduction of the orchid into botanical and nursery gardens and the search for biotechnological approaches to their cultivation *in vitro* are of particular relevance.

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Effect of Zinc Deficiency and Excess on the Antioxidant Enzymes Activity in Barley Seedling Leaves

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Abstract. Zinc (Zn) is an essential microelement in plant nutrition but its high concentrations can be toxic to plants. Either Zn deficiency or its excess negatively affects plant metabolism, in particular due to alterations in cellular redox balance and development of oxidative stress. However, little is known about the effect of Zn deficiency and excess on the activity of antioxidant enzymes and expression of genes encoding them. The aim of this investigation was to study the effect of Zn deficiency and its excess on the intensity of oxidative processes, superoxide dismutase (SOD) and peroxidase (PO) activity, and genes (*HvCu/ZnSOD1* and *HvPRX07*) expression in barley leaves (*Hordeum vulgare* L. cv. Nur). Plants were grown for 7 days at optimal zinc concentration (2 μM), its deficiency (0 μM) and excess (1000 μM). Both stress factors caused similar shoot growth inhibition. However, they both differently influenced the intensity of lipid peroxidation (LPO), total enzyme activity and gene expression. Zn deficiency led to an increase in mRNA content of *HvPRX07* gene, while the activities of PO and SOD were lower compared to those at optimal Zn level. The LPO intensity did not increase. Zn excess caused a significant increase in *HvCu/ZnSOD1* gene expression, and the activity of both enzymes. LPO intensity also increased. This may suggest that under zinc deficiency the inhibition of plant growth is not directly related to the changes of cell redox balance, whereas Zn excess results in an oxidative stress that can cause inhibition of shoot growth.

Keywords: *Hordeum vulgare* L., zinc deficiency, zinc excess, antioxidant enzymes, genes expression.

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Влияние дефицита и избытка цинка на активность антиоксидантных ферментов в листьях ячменя

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Аннотация. Цинк – один из наиболее важных микроэлементов для растений, но в высоких концентрациях он для них токсичен. Поэтому как недостаток металла, так и его избыток приводят к нарушению метаболизма растений, причиной которого может являться изменение редокс-баланса клеток и развитие в них окислительного стресса. Однако данных о влиянии дефицита и избытка цинка на активность компонентов антиоксидантной системы (АОС) относительно немного, а сведений об изменении в этих условиях экспрессии кодирующих их генов практически нет. Вследствие этого целью исследования явилось сравнительное изучение влияния дефицита и избытка цинка на интенсивность окислительных процессов, активность супероксиддисмутазы (СОД) и пероксидазы (ПО) и экспрессию генов *HvCu/ZnSOD1* и *HvPRX07* в листьях ячменя (*Hordeum vulgare* L.). Для этого растения выращивали в течение 7 сут в условиях контролируемой среды при оптимальной концентрации цинка (2 мкМ), его недостатке (0 мкМ) или избытке (1000 мкМ). Обнаружено, что воздействие на проростки обоих стресс-факторов вызывало торможение роста побега, причем почти в равной степени. Однако их влияние на интенсивность перекисного окисления липидов (ПОЛ), общую активность ферментов и экспрессию генов оказалось различным. При дефиците цинка увеличивалось количество транскриптов гена *HvPRX07*, но активность ПО и СОД была ниже, чем при оптимальном уровне металла. Это, однако, не приводило к усилению ПОЛ. При избытке цинка возрастала экспрессия гена *HvCu/ZnSOD1*, увеличивалась активность СОД и ПО, но интенсивность ПОЛ при этом возрастала, свидетельствуя о развитии окислительного стресса. Полученные результаты показывают, что при дефиците цинка задержка роста растений не связана напрямую с нарушением окислительно-восстановительного баланса клеток, тогда как при его избытке (1000 мкМ) окислительный стресс является одной из причин ингибирования роста побега.

Ключевые слова: *Hordeum vulgare* L., дефицит цинка, избыток цинка, антиоксидантные ферменты, экспрессия генов.

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Introduction

Micronutrient bioavailability is an important factor for normal growth, development and high productivity of plants. Zn plays a critical structural role in many proteins, including numerous transcription factors; it is also required as a cofactor in over 300 enzymes. However, excess of Zn can also be toxic to plants. Thus, both Zn deficiency and its excess destroy plant metabolism, inhibit growth and decrease plant productivity. Both stress conditions result in disturbance of cell redox balance and development of oxidative stress (Cakmak, 2000; Singh et al., 2016). Adaptation of plants to either zinc deficiency or zinc excess is mainly associated with the activity of the antioxidant system, including its enzyme components.

Previously, an increase of antioxidant enzymes activity under deficiency (Blasco et al., 2015; Tewari et al., 2019) and excess of zinc (Panda et al., 2003; Li et al., 2013) was reported. It typically corresponds with a decrease of oxidative stress and recovery of physiological processes. However, less is known about expression of genes encoding antioxidant enzymes under stress conditions. As well, few studies have compared the effects of zinc deficiency and its excess on the activity of the antioxidant system, especially in the case of almost identical plant response to these factors related to key physiological processes.

The aim of this study was to compare the effects of zinc deficiency and zinc excess in the growth medium on the intensity of oxidative processes, the activity of antioxidant enzymes and the expression of their encoding genes in barley seedling leaves.

Materials and methods

Seeds of barley (*Hordeum vulgare* L. cv. Nur) were surface sterilized and germinated on filter paper in the dark. One day after, seedlings were transferred to 1.0 L plastic pots with Hoagland–Arnon nutrient solution (pH 6.2 to 6.4) prepared using double-distilled water and high-purity reagents. A chemical analysis of nutrient solutions showed that Zn contamination was ≤ 0.05 μM, which is considered very low for plant growth. Seedlings were cultivated in a growth chamber under a 14-h photoperiod, a photosynthetic photon flux density of 180 μmol m^{-2} s^{-1}, a temperature of 22 °C and relative humidity of 60–70 % on nutrient solutions with optimal zinc content (Zn 2 μM – control), its deficiency (Zn 0 μM) and excess (Zn 1000 μM). Zinc was provided as $ZnSO_4$. Concentrations of 2 and 1000 μM Zn were chosen based on preliminary experiments as optimal and sublethal to this barley cultivar. No Zn was added to the solution to provide deficiency conditions (Zn-deficient medium). Sampling and measurements were performed on the 7th day of exposure with different Zn concentrations. The middle part of the lamina of a fully expanded first leaf was used for the analysis.

Malondialdehyde (MDA) content and antioxidant enzymes activity were determined on a spectrophotometer (Spectrum, Russia) using standard methods as we described earlier (Kaznina et al., 2018). To analyze MDA content, a reaction medium containing 0.25 % solution of thiobarbituric acid (TBA) in 10 % trichloroacetic acid was used. Plant material was homogenized in the reaction medium. The homogenate was aged in a water bath at 95 °C for 30 min, quickly cooled in an ice vessel and centrifuged for 10 min at 10,000 g. The absorbance of the supernatant was measured

at D = 532 and 600 nm. The concentration of TBA-reacting products was calculated using the formula $C_{MDA} = (D_{532} – D_{600})/(\varepsilon \times m)$, where C_{MDA} is the concentration of MDA (μmol g^{-1} wet mass), D_{532} and D_{600} are optical densities of the sample at the appropriate wavelengths, ε is the MDA extinction coefficient equal to 155 $mmol^{-1}$ cm^{-1}, m is the mass of the sample (g).

To determine the content of soluble proteins and the activity of antioxidant enzymes, plant material was homogenized in 0.1 M K/Na-phosphate buffer (pH = 7.8) at 2–4 °C. The homogenate was centrifuged for 20 minutes at 15,000 g and 4 °C. The supernatant was used for the analysis. Soluble protein content was determined by the Bradford method using bovine serum albumin as the standard.

Total activity of superoxide dismutase (SOD) (EC1.15.1.1) was determined based on the ability of SOD to inhibit photochemical reduction of nitrogen tetrazolium to formazan. The amount of the enzyme capable of suppressing the reduction of nitrogen tetrazolium by 50 % was taken as a unit of SOD activity. Peroxidase (PO) activity (EC1.11.1.7) was measured by the increase in optical density at 470 nm resulting from guaiacol oxidation (E = 26.6 $mmol^{-1}$ cm^{-1}) in the presence of hydrogen peroxide.

The expression pattern of *HvCu/ZnSOD1* and *HvPRX07* genes in leaves was monitored by a real-time PCR. Frozen leaf tissues were homogenized with liquid nitrogen. Total RNA was extracted using a TRizol reagent (Evrogen, Moscow, Russia) as instructed by the manufacturer. The total RNA was treated with RNase-free DNase (Syntol, Moscow, Russia) to remove genomic DNA. RNA concentrations and purity of the samples were determined spectrophotometrically (SmartSpecPlus, Bio-Rad, Hercules, USA): samples with A260/A280 ratios within 1.8–2.0 were used for further analysis. The total RNA (1 μg) was reverse-transcribed using a MMLV RT kit (Evrogen) following the supplier's instructions. A real-time quantitative PCR was performed using the iCycler iQ detection system (Bio-Rad). Analyzes were performed using the SYBR Green PCR kit (Evrogen). The PCR conditions consisted in denaturation at 95 °C for 5 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 30 s, and extension at 72 °C for 45 s. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using iCycler iQ. To minimize sample variations, mRNA expression of the target gene was normalized with respect to the expression of the housekeeping gene *actin*. The mRNA content of the target genes was quantified in comparison to the *actin* by the ΔΔCt method (Livak, Schmittgen, 2001). Primers were designed using the Primer Design program and are presented in Table 1.

Table 1. Primers for real-time PCR analysis

Gene	Primers	Nucleotide sequence of the primer 5′–3′	NCBI database accession number
HvActin	direct	ATGTTTTTTTCCAGACG	U21907
	reverse	ATCCAAGCCAACCCAAGT	
HvCu/ZnSOD1	direct	CCTGCCCTTTCCACTCG	HM537232
	reverse	TGTCGTAGGACCGTCATCG	
HvPRX7	direct	TCCACCCTCATCTCCTCCTT	AJ003141
	reverse	ACGGCTTGAACGGTCCTC	

The experiment was carried out using the equipment of the Core Facility in the Karelian Research Center of the Russian Academy of Sciences.

The experiment was conducted using a completely randomized design with 3 replications. The biological replication for different measurements within the control and each treatment group was from 3 to 10 plants, the analytical replication was 3–4-fold. All data are presented as mean ± standard error (SE) from at least three independent replicates. The significance of differences between treatments was calculated by the two-way analysis of variance (ANOVA) using Microsoft Excel 2010. Student's *t*-test was applied to compare statistical significance at the level of $p < 0.05$.

Results

Plants growth

The study showed that both zinc deficiency and its excess during 7 days equally slowed down seedling growth. For instance, in both treatment groups the shoot height was 15 % less than in the control and dry biomass was 7 % less than in the control (Table 2).

MDA content

Content of lipid oxidation products, in particular, MDA, is one of the most informative indicators of oxidative stress in the cell. We found that on the 7th day of plant growth under the conditions of zinc deficiency, there was no increase (as compared to control) in the MDA content in barley leaves, which indicates a low level of reactive oxygen species (ROS) in cells and absence of oxidative stress (Table 3). In contrast, under metal excess, the MDA amount significantly exceeded the control level, indicating an increase of lipid peroxidation intensity.

Antioxidant enzyme activity

We studied SOD, which participates in the superoxide radical detoxification, and PO, which eliminates excess hydrogen peroxide in cells, as

Table 2. The effect of zinc deficiency (0 μM) and excess (1000 μM) on barley (cv. Nur) shoot growth. Mean ± SE, n =10

Parameter	Zinc concentration, μM		
	Zn 2 – control	Zn 0	Zn 1000
Shoot height, cm	18.95±0.25 b	16.19±0.24 a	16.28±0.34 a
Shoot dry biomass, mg	20.37±0.50 b	18.99±0.89 ab	18.99±0.45 a

Note. Different letters denote significant differences at $p < 0.05$ within each row.

Table 3. The effect of zinc deficiency (0 μM) and excess (1000 μM) on malondialdehyde (MDA) content and superoxide dismutase (SOD) and peroxidase (PO) activity in barley (cv. Nur) leaves. Mean ± SE (n = 4)

Parameter	Zinc concentration, μM		
	Zn 2	Zn 0	Zn 1000
MDA content, μM g^{-1} f. w.	2.17±0.10 b	1.83±0.07 a	3.48±0.04 c
SOD activity, conv. un. mg^{-1} protein	2.95±0.28 b	2.00±0.27 a	12.16±0.49 c
PO activity, μmol guaiacol mg^{-1} protein min^{-1}	0.46±0.02 b	0.34±0.03 a	2.35±0.15 c

Note. Different letters denote significant differences at $p < 0.05$ within each row.

key enzymes of antioxidant defense. The results showed that in seedlings growing under zinc deficiency the activity of both enzymes was lower than in the control (Table 3). In contrast, under zinc excess, SOD activity increased as compared to control by 4 times, PO activity increased by 5 times.

The expression pattern of genes

The experiments traced the dynamics of two genes transcripts: *HvCu/ZnSOD1*, encoding one of Cu/ZnSOD isoforms in barley, which is involved in the response to abiotic and biotic stress (Abu-Romman, Shatnawi, 2011), and the *PRX07* gene, the expression of which in barley was previously identified only in response to biotic stress and injury (Kristensen et al., 1999).

The results indicated that after 7 days of seedling growth under zinc deficiency, the amount of *HvCu/ZnSOD1* gene transcripts in leaves remained at the control level, while the amount of *HvPRX07* gene templates was almost 3 times higher (Figure). In contrast, under zinc excess, the mRNA content of *HvCu/ZnSOD1* gene increased notably (almost 5 times compared to the control), while no changes in the *HvPRX07* gene expression were observed.

Discussion

Despite almost equal effect of zinc deficiency and its excess (1000 μM) on barley shoot growth, these stress factors have different influence on the oxidative stress, the antioxidant enzymes activity, and their encoding genes expression.

Zn deficiency

The negative effect of zinc deficiency on the redox balance of plant cells is well known (Sharma et al., 2004; Wang, Jin, 2007; Blasco et al., 2015, etc). The authors conclude that it is largely due to a decrease in the activity of zinc-containing enzymes, for example, Cu/ZnSOD, which neutralizes the superoxide radical in cells. Zinc is also required for mannose-6-phosphate isomerase, a key low-molecular-weight antioxidant in plant cells that is involved in the ascorbate metabolism (Höller et al., 2014).

In the present study, SOD activity was lower under zinc deficiency than in the control, although the level of *HvCu/ZnSOD1* gene expression did not change. It is possible to connect it with the decrease in the activity of the zinc-containing isoform (Cu/Zn SOD), which is believed to make the greatest contribution to the overall enzyme activity (Kliebenstein et al., 1998; Naraikina

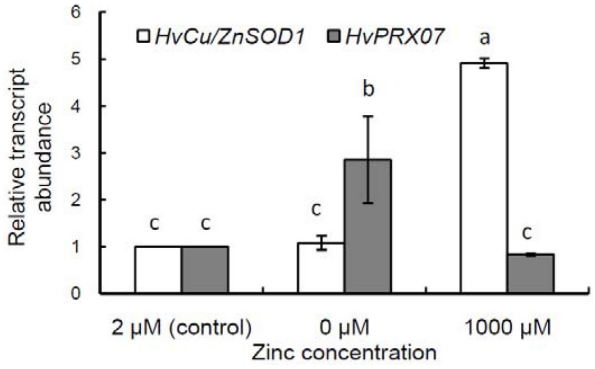

Figure. The effect of zinc deficiency (0 μM) and excess (1000 μM) on *HvCu/ZnSOD1* and *HvPRX7* genes transcription in barley (cv. Nur) leaves. Mean ± SE (n = 3). Different letters denote significant differences at $p < 0.05$

et al., 2014). A decrease in content of hydrogen peroxide produced by SOD could in turn be the cause of the observed decrease in PO activity, as hydrogen peroxide is the substrate for PO. The lower activity of enzymes involved in ROS elimination observed under such conditions could be also explained by occurring changes in physiological processes aimed at reducing the generation of oxygen radicals, for example, by a decrease in the photosynthetic rate (Mattiello et al., 2015).

It is interesting to note that no increase in total PO activity accompanied a significant increase in the amount of *HvPRX07* gene transcripts under zinc deficiency (compared to the control). Possibly, at the initial stages of plant adaptation to a micronutrient deficiency, peroxidase encoded by this gene is involved in other processes, such as regulation of cell growth, lignification of cell walls or auxin metabolism (Cosio, Dunand, 2009).

Concerning the absence of MDA accumulation under zinc deficiency, it can be assumed that at the initial stages of growth seedlings could use the reserves of this trace element from the seed. Moreover, the maintenance of ROS balance under these conditions can be facilitated by an increase in the activity of other antioxidants that do not directly depend on zinc content. For example, a decrease in Cu/ZnSOD activity under Zn deficiency can be partially compensated for by an increase in the activity of other SOD isoforms – Fe-SOD or Mn-SOD, which was shown by some authors (Tewari et al., 2008). In addition, some low molecular weight antioxidants can participate in the superoxide neutralization (Gill, Tuteja, 2010). For instance, under zinc deficiency, it is possible to switch the synthesis of ascorbic acid from the main pathway to an alternative one that does not require the participation of a Zn-containing enzyme (Höller et al., 2014).

Zn excess

Zinc, unlike copper and iron, does not participate in redox reactions, and its accumulation in cells cannot directly lead to an increase in ROS production (Blindauer, Schmid, 2010). However, high concentrations of this metal induce an indirect increase of oxidative stress (Panda et al., 2003; Singh et al., 2016). In this case, an increase in the activity of antioxidant enzymes and non-enzymatic compounds is also observed (Singh et al., 2016). In barley seedlings grown under zinc excess, a significant increase of MDA content was observed, indicating the development of oxidative stress. At the same time, SOD and PO activity increased. The observed lipid peroxidation (LPO) intensification in spite of SOD and PO activation may also be ascribed to exhaustion of the pool of low-molecular antioxidants, for example, carotenoids and reduced glutathione. Glutathione is known to form complexes with heavy metals and is also consumed in the synthesis of phytochelatins (Barrameda-Medina et al., 2014; Bashmakova et al., 2016). It cannot also be excluded that under the stress induced by heavy metals LPO is stimulated through activation of lipoxygenase, the enzyme catalyzing this process (Remans et al., 2012; Liptáková et al., 2013; Barrameda-Medina et al., 2014).

Concerning gene expression, a sharp increase in the amount of *HvCu/ZnSOD* gene transcripts was observed. Apparently, at the initial stages of plant adaptation to a zinc excess, Cu/ZnSOD plays the main role in protecting leaf cells from the superoxide radical, and the increase in SOD activity in this case is likely to be largely associated with the synthesis of enzyme molecules *de novo*. The absence of an increase in the amount of *HvPRX07* gene transcripts in response to zinc excess can be explained by the fact that the PO isoform encoded by this gene may not be involved in plant adaptation to this

stress factor, and the increase in the total PO activity occurs at the expense of other isoforms.

Conclusion

In general, the impact of either zinc deficiency or zinc excess (1000 μM) on barley seedlings within a 7-day period leads to a slowdown in shoot growth; and the effect is almost equal. At the same time, these stress factors influenced differently the intensity of oxidative processes in leaves, SOD and PO activity, and the expression of genes encoding them. Zinc deficiency did not cause an increase in the MDA content in leaves; therefore, inhibition of shoot growth in this treatment group was not associated with the development of oxidative stress in cells. A low activity of both antioxidant enzymes in this case, despite the increase in the mRNA content of *HvPRX07* gene, suggests alternative mechanisms for maintaining the ROS balance in cells. In contrast, under zinc excess, the activity of SOD and PO increased, which corresponded with a sharp increase of *HvCu/ZnSOD1* gene expression. However, a rise in lipid peroxidation intensity observed in this case indicates a violation of pro-antioxidant balance and the development of oxidative stress, which could be one of the causes for shoot growth inhibition under this conditions.

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Redox Reactions in *Hydrocharis morsus-ranae* L. under Industrial Impacts

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Abstract. Aquatic ecosystems are very sensitive to industrial impacts, and, therefore, it is increasingly important to study the mechanisms underlying the tolerance of aquatic organisms to water pollution. Heavy metals (HMs) are among the most common and toxic pollutants of aquatic ecosystems. They have a particularly strong effect on macrophytes, which are in close contact with the aquatic environment and can accumulate metals in considerable quantities. *Hydrocharis morsus-ranae* L. is a floating macrophyte (pleistophyte) with a high capacity for accumulation of HMs. The aim of the present study was to assess the effect of industrial pollution on the redox reactions in *H. morsus-ranae* and to identify the role of low molecular weight antioxidants in adaptation of this macrophyte to unfavorable conditions. A comparative analysis of the physiological and biochemical characteristics of *H. morsus-ranae* from two (reference and impacted) water bodies was carried out. The study revealed an increased level of lipid peroxidation products in the leaves of *H. morsus-ranae* under industrial impact, which indicates oxidative stress. Nevertheless, this floating plant demonstrated fairly high resistance to adverse conditions, due to the synthesis of non-enzymatic antioxidants such as proline and soluble protein thiols. Revealing the response of macrophytes to pollution of water bodies will help predict the state of aquatic ecosystems with an increase in anthropogenic pressure.

Keywords: floating macrophyte, heavy metals, oxidative stress, low molecular weight antioxidants.

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Редокс-реакции у *Hydrocharis morsus-ranae* L. в условиях техногенной нагрузки

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Аннотация. Водные экосистемы характеризуются высокой чувствительностью к техногенным нагрузкам, поэтому все более актуальным является изучение механизмов устойчивости гидробионтов к загрязнению водных объектов. Тяжелые металлы (ТМ) относятся к наиболее распространенным и токсичным поллютантам гидроэкосистем. Особенно сильное воздействие они оказывают на макрофиты, которые контактируют с водной средой и могут накапливать металлы в значительных количествах. *Hydrocharis morsus-ranae* L. относится к плавающим макрофитам (плейстофитам), обладающим высокой аккумулятивной способностью по отношению к ТМ. Цель исследования – оценка влияния техногенного загрязнения на редокс-реакции у *H. morsus-ranae,* а также выявление роли низкомолекулярных антиоксидантов в его адаптации к неблагоприятным условиям. Проведен сравнительный анализ физиолого-биохимических характеристик *H. morsus-ranae* из двух водных объектов (фон и импакт). Исследование определило повышенный уровень содержания продуктов перекисного окисления липидов в листьях *H. morsus-ranae* в условиях техногенного воздействия, что свидетельствует об окислительном стрессе. Тем не менее этот макрофит продемонстрировал достаточно высокую устойчивость к неблагоприятным условиям, что стало возможным благодаря синтезу таких неэнзиматических антиоксидантов, как пролин и растворимые белковые тиолы. Выявление ответных реакций макрофитов на загрязнение водных объектов будет способствовать прогнозированию состояния гидроценозов при усилении антропогенного прессинга.

Ключевые слова: плавающий макрофит, тяжелые металлы, окислительный стресс, низкомолекулярные антиоксиданты.

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Introduction

Hydrocharis morsus-ranae L. (Hydrocharitaceae Juss. family) is one of the widespread floating macrophytes (pleistophytes). Macrophytes play an important role in production processes, transformation of organic compounds, and biogeochemical cycles of elements in water bodies. Aquatic ecosystems are very sensitive to industrial impacts. Therefore, it is increasingly important to study the mechanisms responsible for the tolerance of macrophytes to environmental pollution (Polechońska et al., 2017; Gałczyńska et al., 2019).

Heavy metals (HMs) are among the most common and dangerous pollutants of the hydrosphere (Kabata-Pendias, Mukherjee, 2007). As is known, with an increased HM level in the habitat, aquatic plants can accumulate HMs in rather high amounts, which are sometimes several thousand times greater than the HM content in the surface waters (Borisova et al., 2016; Gałczyńska et al., 2019).

The high ability of *H. morsus-ranae* to accumulate such metals as Mn, Fe, Co, Ni, Zn, and Cu has already been noted (Brekhovskikh et al., 2009; Polechońska, Samecka-Cymerman, 2016). Polechońska and Dambiec (2014) reported that the contents of Mn, Fe, and Cu in the leaves of *H. morsus-ranae* exceeded the average levels observed for other floating macrophytes, even in cases of low metal content in water bodies. At the same time, the average concentrations of Zn, Mn, and Fe exceeded physiological values and corresponded to the level of toxicity (Polechońska, Dambiec, 2014).

The study of the HM accumulation by *H. morsus-ranae* during the growing season showed that the maximal contents of most HMs in plants and the highest bioconcentration factors were noted in June. It has been suggested that the increased content of metals in the floating macrophyte at the beginning of the growing season may be associated with the contact of hibernating buds with contaminated sediments during the winter (Polechońska et al., 2017).

The HM bioaccumulation in *H. morsus-ranae* shoots depends on the type of industrial activities. The highest levels of Cd and Co were found near plants producing organic compounds and the automotive industries; elevated concentrations of Cr, Cu, and Pb were noted near thermal power plant and former ferrochrome industry. At the same time, the concentrations of alkali metals, Co, and Fe in *H. morsus-ranae* were higher than in other aquatic plants regardless of

their amounts in the environment (Polechońska, Samecka-Cymerman, 2016).

HM concentrations in the *H. morsus-ranae* leaves were ranked as follows: Mn > Fe > Zn > > Cu > Hg. Based on the bioaccumulation and translocation factors, it was concluded that *H. morsus-ranae* is an accumulator of Co, Cr, Cu, Fe, K, Mn, Ni, Pb, and Zn (Polechońska, Samecka-Cymerman, 2016). Moreover, the roots accumulate more HMs than the leaves (Gałczyńska et al., 2019). Significant positive correlations were also found between the contents of Zn, Fe, and Hg in *H. morsus-ranae* and in water, which indicates the possibility of using this floating macrophyte in the biomonitoring of water pollution (Brekhovskikh et al., 2009).

Thus, a lot of data have been reported on the high tolerance of *H. morsus-ranae* to environmental pollution with HMs and its accumulative abilities. However, there is only fragmentary information on the adaptive physiological and biochemical mechanisms contributing to the growth and vitality of this floating macrophyte even under considerable industrial impacts.

The aim of the study was to assess the effect of industrial pollution on redox reactions in *H. morsus-ranae* and identify the role of low molecular weight antioxidants in adaptation of this species to unfavorable conditions.

Materials and methods

H. morsus-ranae is a cosmopolitan perennial plant with numerous roots hanging down into the water column. The leaf blade is rounded; the aerenchyma is not developed. The plant has a high growth rate and vegetative reproduction, prefers water bodies with an average trophic status, grows best in low-flow habitats such as swamps, river backwaters, lakes, and reservoirs (Polechońska, Dambiec, 2014; Polechońska, Samecka-Cymerman, 2016).

Sampling of surface waters, sediments, and plant material was carried out in the Chelyabinsk Region (South Ural, Russia) in July 2018–2019. Samples were collected from two sites: the reference site (Site 1) and the impacted site (Site 2). The coastal water of the Irtyash Lake (55°52'22" N 60°42'16" E) was used as the reference site (Table 1). This reservoir lake is located in the Kaslinsky district of the Chelyabinsk Region. According to its primary productivity and nutrient content, the Irtyash Lake is a mesotrophic water body.

The Egoza River backwater (55°44'03" N 60°30'57" E) near the town of Kyshtym was used as an impacted site (Table 1). There are several industrial facilities in Kyshtym, and due to their activities, various pollutants, including HMs, enter the river. In addition, the increased contents of many HMs are associated with the presence of serpentinite rocks, which contain

Table 1. The pH value, specific electrical conductivity, and total index of toxic load in the study sites

Parameter	Site 1	Site 2
pH value of water	6.9 ± 0.1	6.8 ± 0.2
Specific electrical conductivity of water, μS/cm	392.7 ± 33.2	551.3 ± 35.3*
Si (water)	1.0	3.4
Si (sediments)	1.0	2.0

Data presented as Mean ± SE; asterisk (*) indicates significant differences between the study sites at $p < 0.05$.

high concentrations of Ni, Fe, Cr, and Co (Tripti et al., 2021).

The pH and specific electrical conductivity of the water were measured using a pH meter/ conductometer (Hanna Instruments, Germany).

To determine the HM content, water and sediment samples were taken at four points of each site and mixed to prepare a composite sample. The content of metals was determined in three replicates by inductively coupled plasma atomic emission spectrometry (iCAP 6500 Duo, Thermo Fisher, U.S.A.) after wet digesting with 70 % HNO_3. As an integrated indicator of water and sediment pollution, the total index of toxic load (*Si*) was used, which was calculated using the formula (Bezel et al., 1998):

$$Si = (1/n)\sum_{i=1}^{n}\left(\frac{C_i}{C_{reference}}\right) \quad (1)$$

where, C_i is the concentration of metal in water/ sediments of the impacted site; $C_{reference}$ is the concentration of metal in the water/sediments of the reference site; n is the number of metals studied.

Physiological and biochemical characteristics such as the rates of lipid peroxidation, content of carotenoids, free proline, phenolic compounds, soluble thiols and proteins were measured in the leaves of *H. morsus-ranae* spectrophotometrically on a PD-303UV (APEL, Japan), according to standard methods.

The rate of lipid peroxidation was determined by the reaction of malonic dialdehyde (MDA) with thiobarbituric acid (TBA). Absorbance was measured at 532 and 600 nm (Heath, Packer, 1968). The contents of photosynthetic pigments (chlorophylls and carotenoids) were measured at 470, 647, and 663 nm after extraction in 80 % acetone. The carotenoid content was calculated according to Lichtenthaler (1987). Free proline content was determined after extraction in hot water (95 °C) and boiling in a water bath for 20 min (Kalinkina et al., 1990). For staining, a mixture of ninhydrin reagent with glacial acetic acid (1:1) was used; absorbance was measured at 520 nm. The total content of soluble phenolic compounds was determined at 760 nm using the Folin-Chiocalteu reagent after preliminary extraction with 70 % ethanol solution for 24 hours. Gallic acid was used as a standard (Singleton et al., 1999). The extraction and determination of protein and non-protein thiols were performed as described by Borisova et al. (2016). The total content of soluble thiols was determined after reaction with Elman's reagent (5.5'-dithiobis (2-nitrobenzoic) acid) at 412 nm. The content of protein thiols was calculated by subtracting the amount of non-protein thiols previously obtained by precipitation of proteins with 50 % trichloroacetic acid from the total soluble fraction. Reduced glutathione was used as a standard (Borisova et al., 2016). The content of soluble protein was determined at a wavelength of 595 nm according to Bradford (1976). Bovine serum albumin was used as a standard. Determination of physiological and biochemical parameters was carried out in 4 biological and 3 analytical replicates (n = 12). All parameters were measured on fresh plant material and then calculated as per one g of dry weight (DW).

The significance of differences was assessed using the nonparametric Mann–Whitney test at a significance level of $p < 0.05$. The tables and figures show the mean values (Mean) and their standard errors (SE); asterisks indicate significant differences between the study sites.

Results and discussion

The study sites did not differ significantly in water pH, while the specific electrical conductivity of the surface waters in the impacted site (the Egoza River) was 1.4 times higher than in the reference one (the Irtyash Lake, Table 1).

The total index of toxic load at the impacted site (Site 2) was calculated from the contents of four metals (Fe, Zn, Mn, and Ni). The toxic load for water was 1.7 times higher than for sediments. The average value of this index for surface waters and sediments was 2.7 (Table 1).

The maximum difference between sites in the contents of metals was found for Fe (5.6 times), Zn (2.4 times), Mn (2.2 times), Ni (3.0 times), and Co (4.0 times) in water, and for Ni, Pb, and Zn in sediments (4.2, 2.7, and 1.6 times, respectively) (Table 2).

Most of the concentrations of metals in water exceeded the maximum permissible concentrations (MPC) for fishery water bodies (Water quality standards …, 2016). The highest excess of MPC was noted for mercury in the water of Site 2 (66 times), while for zinc, iron, and manganese it was 5 times higher on average. Among the metals studied, the most toxic to plant organisms are Hg, Co, Ni, Cu, and Pb (Kabata-Pendias, Mukherjee, 2007). Their contents in Site 2 were higher both in water and in sediments. The exception was Pb, whose concentration in the water of the impacted site was slightly lower than in the reference one (Table 2).

As is well known, an excess of HMs in the habitat causes inhibition of photosynthesis in plants, impaired transport of assimilates and mineral nutrition, growth inhibition, and changes in the water and hormonal status. Increased concentrations of HMs (especially of such redox-active ones as copper, iron, and manganese) can promote the generation of reactive oxygen species (ROS) (Blokhina et al., 2003; Gill, Tuteja, 2010). Lipid peroxidation, whose rates are assessed by the MDA content, is a reaction indicating damage to cell membranes (Blokhina et al., 2003; Sharova, 2016).

An assessment of the lipid peroxidation rate in the leaves of *H. morsus-ranae* showed that the MDA content in plants in Site 2 was 1.2 times higher than in Site 1 (Fig. 1a). An increase in the MDA content in the plants under anthropogenic load indicates the development of oxidative stress, despite the fact that most metals accumulate in the *H. morsus-ranae* roots.

The concentration of ROS formed in the cell is maintained at a sufficiently low level by a multicomponent antioxidant defense system, the state of which largely determines plant resistance to stress (Blokhina et al., 2003; Sharova, 2016). ROS and antioxidants interacting with them are presently considered as redox-active molecules, which are the main participants in redox processes (Pradedova et al., 2017). As is well known, ROS

Table 2. The contents of heavy metals in water and sediments of the study water bodies

Metal	Metal content in water, μg/L		Metal content in sediments, mg/kg	
	Site 1	Site 2	Site 1	Site 2
Fe	94.27 ± 8.87	527.90 ± 39.81*	26505.48 ± 1429.05	28602.91 ± 1293.90
Zn	25.42 ± 2.76	60.06 ± 1.36*	72.15 ± 2.51	115.19 ± 9.75*
Mn	19.27 ± 0.77	43.40 ± 1.50*	614.02 ± 3.06	768.17 ± 14.47*
Ni	9.16 ± 0.55	27.12 ± 2.08*	32.40 ± 1.05	136.90 ± 7.85*
Cu	19.53 ± 1.75	23.45 ± 0.19	60.06 ± 1.25	76.92 ± 1.35*
Co	0.50 ± 0.01	2.06 ± 0.16*	8.33 ± 1.65	10.94 ± 0.68
Hg	0.40 ± 0.01	0.66 ± 0.09*	13.44 ± 0.64	14.38 ± 0.33
Pb	5.41 ± 0.16	3.04 ± 0.40*	45.34 ± 1.26	120.46 ± 8.48*

Data presented as Mean ± SE; asterisk (*) indicates significant differences between the study sites at $p < 0.05$.

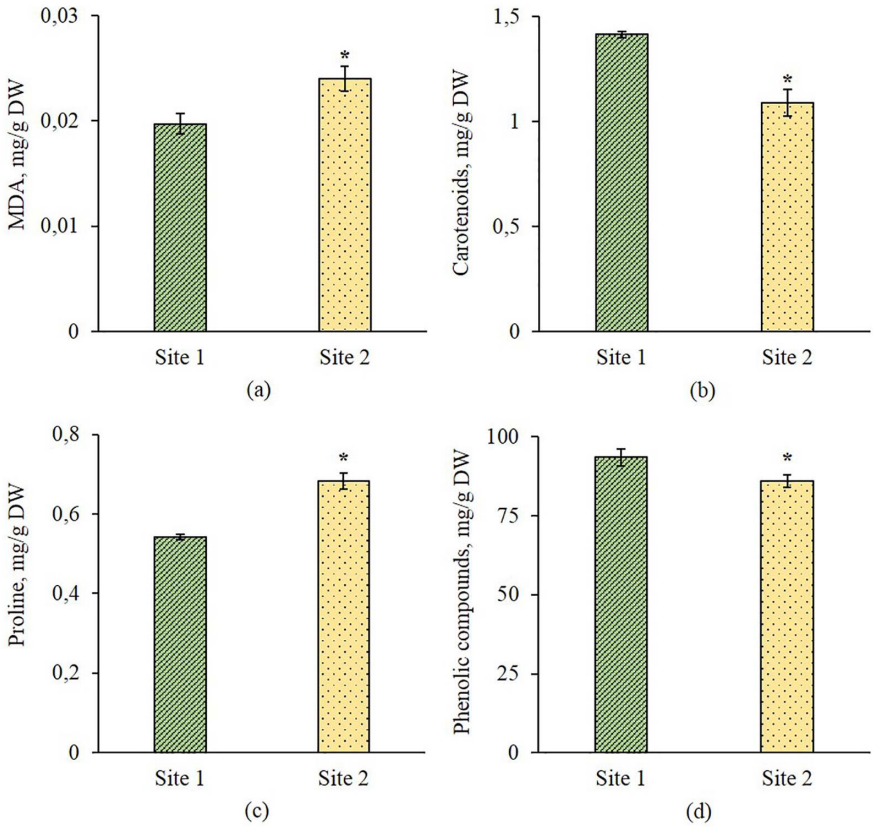

Fig. 1. Contents of malondialdehyde (a), carotenoids (b), free proline (c), and soluble phenolic compounds (d) in the leaves of *H. morsus-ranae*. Data presented as Mean ± SE; asterisk (*) indicates significant differences between the study sites at $p < 0.05$

in plants are involved in the regulation of many vital processes. At the same time, antioxidants play a leading role in the understanding of the physiological functions of ROS (Sharova, 2016; Pradedova et al., 2017).

Carotenoids are the multifunctional compounds of plants. Not only do they take part in the absorption of light energy and the protection of green pigments from photodegradation, but they also have antioxidant activity (Strzalka et al., 2003). The content of carotenoids in *H. morsus-ranae* in Site 2 was significantly (1.4 times) lower than in Site 1 (Fig. 1b). A decrease in the carotenoid content in a habitat contaminated with metals was also noted in our previous study of the helophyte *Typha latifolia* L. (Maleva et al., 2019). Carotenoid molecules have double bonds, and they are oxidized when interacting with ROS (Strzalka et al., 2003). Thus, a decrease in the content of carotenoids in plants from contaminated habitats is apparently a consequence of their oxidative degradation.

Proline is a proteinogenic heterocyclic amino acid, which plays an important role in the plant cells. It is involved not only in osmoregulation, but also in the stabilization of proteins, membranes, and subcellular structures. Proline is also able to chelate HMs, maintain cellular redox potential, and participate in ROS neutralization (Hare, Cress, 1997; Sharova, 2016). The free proline content in *H. morsus-ranae* from Site 2 increased by 26 % compared to Site 1 (Fig. 1c), which indicates its active role in the adaptation of the macrophyte to the environmental pollution.

Many phenolic compounds are known to have antioxidant properties. Due to OH groups, phenols can participate in the detoxification of ROS (Sharova, 2016). Phenolic substances readily interact with reactive oxygen species. Initially, they are oxidized to phenoxyl radicals, the further oxidation of which leads to the formation of quinones. They can also chelate HMs and stabilize membranes, which limits the diffusion of free radicals and reduces the rate of lipid peroxidation (Michalak, 2006). As a rule, under stress, the synthesis of phenolic compounds is enhanced (Pourcel et al., 2007; Sharova, 2016). However, the present study demonstrated that the content of soluble phenolic components in the leaves of *H. morsus-ranae* in Site 2 was 17 % lower than in Site 1 (Fig. 1d). It can be assumed that, as a result of the high level of toxic load, the rate of destruction of phenols was higher compared to its synthesis reactions. Interestingly, our previous study of the helophyte plant *Typha latifolia* L. revealed an opposite trend for phenolic compounds (Maleva et al., 2019). That species demonstrated higher tolerance to industrial pollution than *H. morsus-ranae*. At the same time, they share similar trends in changes of the contents of other non-enzymatic antioxidants, which indicates a major role of these secondary metabolites in the formation of tolerance to HMs.

Compounds containing SH-groups (thiols), which can be divided into protein and non-protein ones, play an important part in the antioxidant protection of plants. Thiols can both bind HMs and act as antioxidants, participating in the neutralization of ROS formed during oxidative stress (Cobbett, 2000; Sharova, 2016). A previous study demonstrated that the contents of soluble thiols in different species of aquatic plants correlated with the accumulation of HMs (Borisova et al., 2016). The present study showed that the content of soluble thiols in the contaminated site (Site 2) was significantly (1.2 times) higher than in the reference site (Fig. 2a). Moreover, protein thiols prevailed over non-protein ones: their content was about 86 % of the total amount of soluble thiols.

Many proteins are involved in the antioxidant defense system of plants. They are capable of both directly chelating HMs and acting as enzymes, catalyzing the reactions of ROS neutralization (Kulaeva, Tsyganov, 2011). Determination of the total content of soluble protein revealed an increase in its amount in plants from Site 2 compared to the reference ones (by an average of 24 %, Fig. 2b).

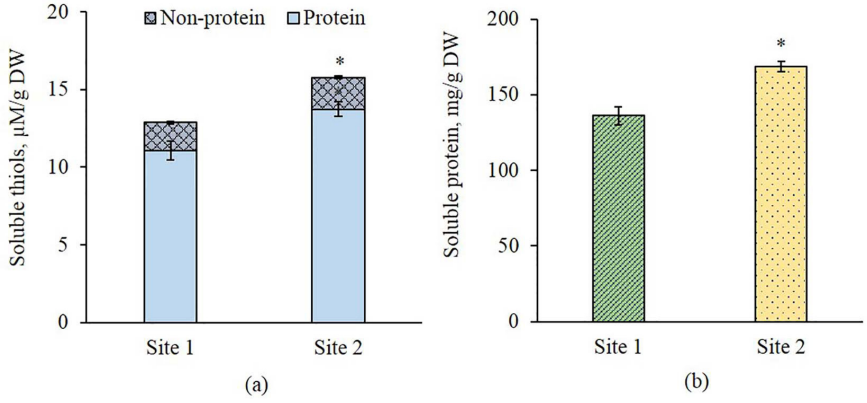

Fig. 2. Contents of soluble thiols (a) and protein (b) in the leaves of *H. morsus-ranae*. Data presented as Mean ± SE; asterisk (*) indicates significant differences between the study sites at $p < 0.05$.

Thus, the study of the *H. morsus-ranae* redox reactions to environmental pollution showed that the development of oxidative stress was accompanied by the accumulation of proline, soluble thiols, and proteins in cells, suggesting activation of their synthesis under anthropogenic load.

Conclusion

Comparative analysis of the redox reactions of the floating macrophyte *H. morsus-ranae* from natural habitats with different levels of industrial impact revealed some adaptive features of the plant. The increased level of lipid peroxidation products in the leaves of *H. morsus-ranae* from the impacted site indicates oxidative stress. At the same time, the macrophyte demonstrated a fairly high resistance to the pollution of the habitat by heavy metals, which was probably due to the increased synthesis of such non-enzymatic antioxidants as proline and soluble protein thiols.

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Comparative Evaluation of Prooxidant/Antioxidant Balance in Seed Progeny of *Plantago major* L. from Radioactively and Chemically Contaminated Areas

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Abstract. There are many studies addressing plant responses to radioactive and chemical contamination of soils, but few works have been devoted to comparison of biological effects in the areas affected by these human-induced factors. Ionizing radiation and heavy metals have different mechanisms of interaction with biota. Both factors, however, are capable of increasing the generation of reactive oxygen species, which cause enzyme malfunction and cell structure damage. The efficiency of antioxidant systems plays an important role in plant resistance to these impacts. The present study offers a comparative evaluation of prooxidant/antioxidant balance in seed progeny of *Plantago major* L. growing in the East Ural Radioactive Trace (EURT), in the zone affected by operation of the Karabash Copper Smelter (KCS), and in the reference sites. Lipid peroxidation was assessed by determining malondialdehyde. Evaluation of the antioxidant system was based on the activities of superoxide dismutase, catalase, and total peroxidase, and on the content of low-molecular-weight antioxidants. The study showed that the prooxidant and antioxidant statuses of seed progeny of *P. major* from the contaminated sites were different from the reference samples and from each other. The pooled EURT sample exhibited a prooxidant shift relative to the reference samples, i. e. not only malondialdehyde but also activities of superoxide dismutase and catalase and the content of low-molecular-weight antioxidants were higher than in the reference samples. Malondialdehyde content in seedlings from the KCS zone did not differ from the reference values; superoxide dismutase and catalase activities were decreased whereas peroxidase activity was higher compared to the activities of these enzymes in the reference samples. Thus, the differences in the plant adaptive responses to ionizing radiation and heavy metals are caused by the dissimilarities in the induction of reactive oxygen species.

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Keywords: *Plantago major*, oxidative stress, antioxidant protection system, radioactive contamination, heavy metal contamination.

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Сравнительная оценка про- и антиоксидантного статуса семенного потомства *Plantago major* L. из зон радиоактивного и химического загрязнения

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Аннотация. Ответные реакции растений на действие радиации и тяжелых металлов изучены в многочисленных исследованиях, но работ по сравнению биологических эффектов в зонах влияния этих техногенных факторов известно немного. Радиация и тяжелые металлы имеют разные механизмы взаимодействия с биотой, но оба фактора способны усиливать образование активных форм кислорода, вызывающих нарушение работы ферментов и повреждение клеточных структур, поэтому важную роль в устойчивости растений к этим воздействиям играет эффективность работы антиоксидантных систем. Дана сравнительная оценка про- и антиоксидантного статуса семенного потомства *Plantago major* L. из популяций, произрастающих в головной части Восточно-Уральского радиоактивного следа (ВУРС) и в зоне влияния Карабашского медеплавильного завода (КМЗ), а также на фоновых территориях. Интенсивность процессов перекисного окисления липидов определяли по концентрации малонового диальдегида. Работу антиоксидантной системы оценивали по активности трех ферментов: супероксиддисмутазы, каталазы и общей пероксидазной активности, а также по суммарному содержанию низкомолекулярных антиоксидантов. Установлено, что про- и антиоксидантные статусы семенного потомства подорожника из техногенных зон отличны от фоновых выборок и различаются между собой. У проростков из зоны ВУРСа зафиксирован прооксидантный сдвиг, т. е. при высоком содержании малонового диальдегида была повышена активность супероксиддисмутазы и каталазы, а также содержание низкомолекулярных антиоксидантов по сравнению с фоновыми растениями. У проростков из зоны КМЗ содержание малонового диальдегида не отличалось от фонового, активности супероксиддисмутазы и каталазы были снижены, а пероксидазы повышена. Таким образом, специфика адаптивных

ответов растений на действие радиации и тяжелых металлов формируется за счет различий в индукции активных форм кислорода.

Ключевые слова: *Plantago major*, окислительный стресс, система антиоксидантной защиты, радиоактивное загрязнение, загрязнение тяжелыми металлами.

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Introduction

A substantial amount of research has been focused on the effects of ionizing radiation (Esnault et al., 2010; Møller, Mousseau, 2015; Caplin, Willey, 2018) and heavy metals (Titov et al., 2014; Zvereva et al., 2010; Sharma, Agrawal, 2005; Fischer et al., 2013) on natural plant populations, but few studies have been devoted to comparison of biological effects in plants growing in the areas affected by different anthropogenic factors (Pozolotina et al., 2012; 2016). Ionizing radiation and heavy metals differ in their physical nature and have different mechanisms of interaction with biota at the molecular level. However, both factors can increase the generation of reactive oxygen species (ROS) and induce oxidative stress; therefore, the efficiency of the antioxidant system plays an important role in plant resistance to anthropogenic stressors (Khramova et al., 2006; Sharma et al., 2012; Morozova et al., 2016; Volkova et al., 2017; Gudkov et al., 2019). Thus, the adaptive response of plants may vary depending on the type of stressor. The aim of this study was to assess and compare the prooxidant/antioxidant status in seed progeny of *Plantago major* L. plants that have been growing for a long time in radioactively or chemically contaminated sites and in the reference areas.

Materials and methods

The greater plantain (*P. major*) is a perennial herbaceous polycarpic plant of the Plantaginaceae family. This species is diploid ($2n = 12$), reproduces mainly by seeds; humans unintentionally participate in the spread of plants (anthropochory) (Ontogenetic atlas of medicinal plants, 1997). The study was carried out in regions with different types of industry-induced pollution: the East Ural Radioactive Trace (EURT), the area affected by the Karabash Copper Smelter (KCS) emissions, and reference areas.

The EURT was formed because of an accident at the «Mayak» Production Association on September 29, 1957: the explosion of the tank with radioactive waste. The main pollutant is ^{90}Sr, whose half-life is 28.8 years (Nikipelov et al., 1990). Even 60 years after the EURT formation, densities of soil contamination by ^{90}Sr along the central axis of the trace exceed the background level by 2–4 orders of magnitude (Molchanova et al., 2014). Three sites were selected along the EURT central axis (Fig. 1). Absorbed dose rates for *P. major* maternal plants were calculated using the ERICA Tool and taking into account the contributions of ^{90}Sr, ^{137}Cs, and ^{239,240}Pu and the natural background radiation (Karimullina et al., 2018). Absorbed dose rates within the EURT ranged from 19.1 to 157.1 μGy/h (background value = 0.109 μGy/h), which

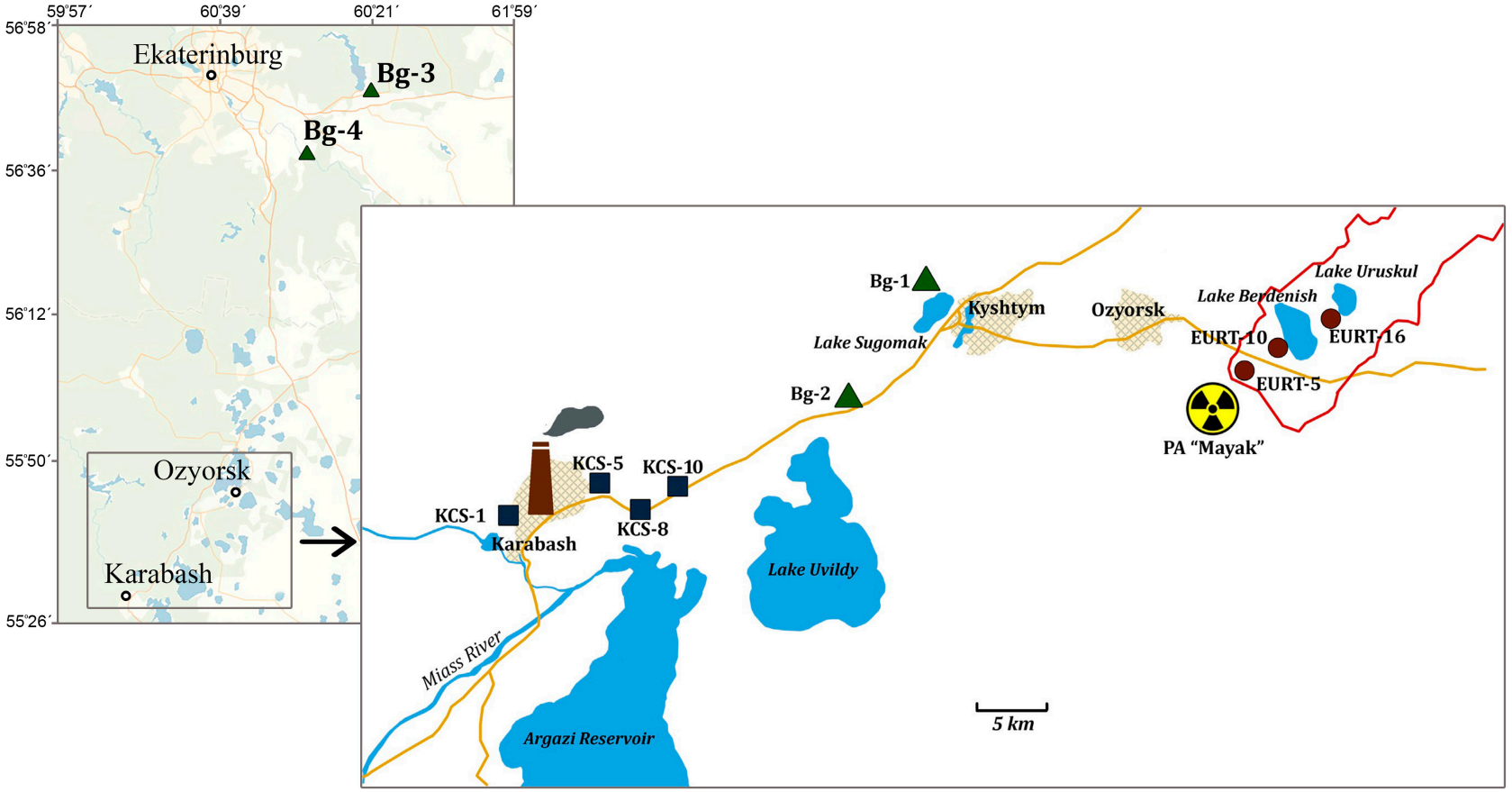

Fig. 1. Schematic map of the study region. The names of affected sites indexed by the distance from the pollution source

corresponded to the low-dose range for plant organisms (Garnier-Laplace et al., 2013).

The Karabash Copper Smelter was put into operation in 1910, with no equipment for treatment of atmospheric emissions and wastewater. Emissions of pollutants into the atmosphere were at their maximum levels in the 1960–1970s. The main components of the smelter emissions were sulfur dioxide and dust containing heavy metal (HM) particles (Stepanov et al., 1992). The KCS operations were suspended from 1989 to 1997, and emissions have considerably decreased after reconstruction (Stepanov et al., 1992; Chernen'kova, 2002). Nevertheless, the amount of heavy metals (Zn, Cu, Pb, Cd) accumulated in the soils is still very high, exceeding the background level by at least one order of magnitude. The soil in the contaminated area has increased acidity (pH_{H2O} = 5.8, with the background pH = 6.4) (Stepanov et al., 1992; Smorkalov, Vorobeichik, 2011). Four sites were selected in the chemically contaminated area at different distances from the KCS (Fig. 1). The toxic load indices at the KCS study sites were higher than the average background value by factors of 5.2–41.8 (Shimalina et al., 2017).

Reference sites were located outside the radioactive and chemically polluted areas in the Sverdlovsk and Chelyabinsk Regions (Fig. 1); detailed descriptions of test sites are given in (Shimalina et al., 2017; 2018). All sampling sites were located at the sides of rarely used country roads, on which transects from 0.5 to 1 km in length were laid. A pooled sample of seeds from 40–50 plants was collected from each site. *P. major* seeds were cultivated for 21 days at 24 °C and 12-hour photoperiod in filter paper rolls placed in glass vessels with distilled water. The grown seedlings were used for biochemical analyses. The prooxidant status was assessed by the concentration of the secondary products of lipid peroxidation (LPO), the main of which is malondialdehyde (MDA). The MDA content was determined using a reaction medium consisting of a solution of trichloroacetic and thiobarbituric acids; absorbance measurements were performed at 532 nm reference wavelength (Buege, Aust, 1978). Antioxidant status was evaluated by the activity of three enzymes: superoxide dismutase (SOD, EC1.15.1.1), catalase (CAT, EC1.11.1.6), and peroxidase (POX, EC1.11.1.7). Dried seedlings were homogenized in a Tris-HCl pH 7.4 buffer solution. The homogenate was centrifuged for 10 min (t = +4 ºC). The SOD activity was assessed at 560 nm by inhibition of the photochemical formation of a colored reduced product of nitro blue tetrazolium with the participation of riboflavin and *L*-methionine (Giannopolitis, Ries, 1977). The CAT activity was measured at 410 nm by the intensity of the yellow color formed in the reaction of hydrogen peroxide with molybdenum salts (Góth, 1991). The POX activity was determined at 610 nm by the decrease in the color intensity of the reaction mixture upon indigo carmine oxidation by hydrogen peroxide (Popov, Neĭkovska, 1971). Soluble protein content determination was based on the qualitative reaction with the Coomassie Brilliant Blue G250 dye (Kruger, 2009). Measurements of MDA and protein amounts and enzyme activities were carried out using a SpectraMax Plus 384 microplate spectrophotometer (Molecular Devices, U.S.A.). Also, total low-molecular-weight antioxidants (LMWA) in seedlings were determined. The determination of total LMWA was based on LMWA oxidation with iron chloride (III) (Ermakov et al., 1987); absorbance measurements were performed using a DU-650 spectrophotometer (Beckman Coulter, U.S.A.) at 510 nm reference wavelength.

Data analysis was performed using the criteria of nonparametric statistics (Mann-Whitney *U*-test, *H*-test – Kruskal-Wallis rank analysis of variance, Dunn test). For multivariate comparison of biochemical data, the principal

component method was used. Data analysis was performed with the STATISTICA 10.0 (StatSoft Inc., 2011) software.

Results

Differences in the biochemical status parameters of seedlings were insignificant between three samples from the EURT: MDA ($H_{(2;21)}$ = 3.17, p = 0.205); SOD ($H_{(2;21)}$ = 3.36, p = 0.186); CAT ($H_{(2;21)}$ = 2.42, p = 0.297); POX ($H_{(2;21)}$ = 2.42, p = 0.297). Similarly, there were no differences between four local populations from the KCS: MDA ($H_{(3;25)}$ = 2.72, p = 0.436); SOD ($H_{(3;25)}$ = 5.52, p = 0.137); CAT ($H_{(3;25)}$ = 5.24, p = 0.155); POX ($H_{(3;25)}$ = 5.67, p = 0.129). No significant differences were found between the parameters of the reference samples of *P. major*: MDA ($H_{(2;15)}$ = 3.24, p = 0.198); SOD ($H_{(2;15)}$ = 1.38, p = 0.501); CAT ($H_{(2;15)}$ = 5.27, p = 0.071); POX ($H_{(2;15)}$ = 2.49, p = 0.287). Therefore, for further analysis, the samples were pooled within the EURT, KCS, and reference sites.

The MDA content in *P. major* seedlings from the EURT sites was significantly increased compared to the reference values (228 % of the reference values; *U*-test, n = 15–21, $p < 0.001$). In the KCS samples, the MDA content did not differ from the reference values (*U*-test, n = 15–25, p = 0.276). Comparison of the EURT and KCS samples revealed significant differences in the MDA content (*U*-test, n = 21–25, p = 0.016) and SOD, CAT, and POX activities (*U*-test, n = 21–25, $p < 0.001$).

P. major samples from the EURT sites significantly differed from the reference ones in enzyme activity: the SOD and CAT activities were increased (270 % of the activity in the reference samples; *U*-test, $p < 0.001$, and 199 %; *U*-test, p = 0.040, respectively) and POX activity, on the contrary, was lower than in the reference samples (44 %; *U*-test, p = 0.011). The enzyme activity of the KCS samples also significantly differed from the enzyme activity of the reference samples. Seedlings from the KCS sites showed a decreased SOD activity (60 % of the activity in the reference samples; *U*-test, p = 0.029), a decreased CAT activity (33 %; *U*-test, $p < 0.001$), and a higher POX activity (122 %; *U*-test, $p < 0.001$). Thus, in seedlings from the EURT sites, MDA content and SOD and CAT activities were higher and POX activity was lower compared to the samples from both the KCS and reference sites.

Multivariate comparison of biochemical data by the method of principal components (Fig. 2) showed that point projections of samples from contaminated and reference sites partially overlapped with each other for the first principal component, PC-1. This component explained 72.7 % of intergroup variance, its value was based on the correlation with the activity of SOD (β = 0.98), CAT (β = 0.99), and POX (β = –0.98). The EURT samples were more isolated for PC-2 (it explained 25.5 % of variance), where the main contribution to intergroup variability was made by the MDA value (β = 0.99).

The prooxidant/antioxidant index (PAI), calculated as the MDA/(SOD+CAT+POX) ratio, was used as the integrated indicator reflecting the general level of functioning of the antioxidant system components. Comparison of PAI values (Fig. 3) suggested the presence of a significant prooxidant shift in the EURT samples relative to the reference (331 %, *U*-test, n = 15–21, $p < 0.001$) and KCS samples (510 %, *U*-test, n = 15–25, $p < 0.001$). The results indicated that under these conditions of enzymatic protection, which deactivates reactive oxygen species, the rate of accumulation of secondary lipid peroxidation products in the affected local populations of the EURT was 3 and 5 times higher than in the reference and KCS samples, respectively. Another type of the prooxidant/antioxidant ratio was noted in seedlings from the KCS sites: no significant differences were found between the

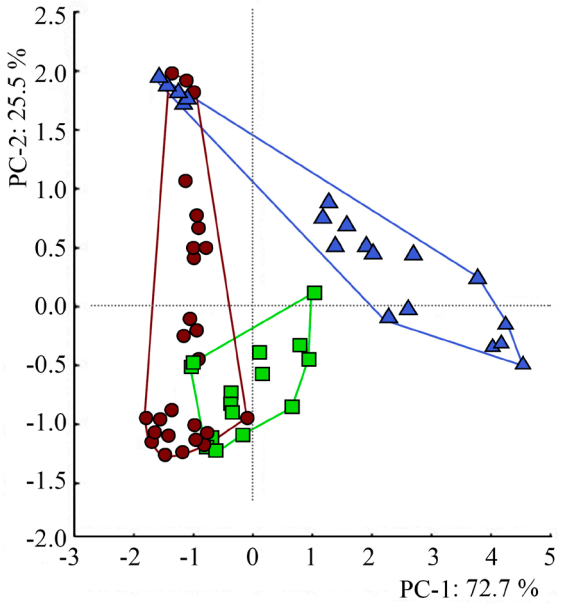

Fig. 2. Arrangement of reference, KCS and EURT samples in the plane of the two principal components formed by biochemical parameters: PC-1 – antioxidant enzymes; PC-2 – MDA; green squares – reference samples; red circles – KCS; blue triangles – EURT

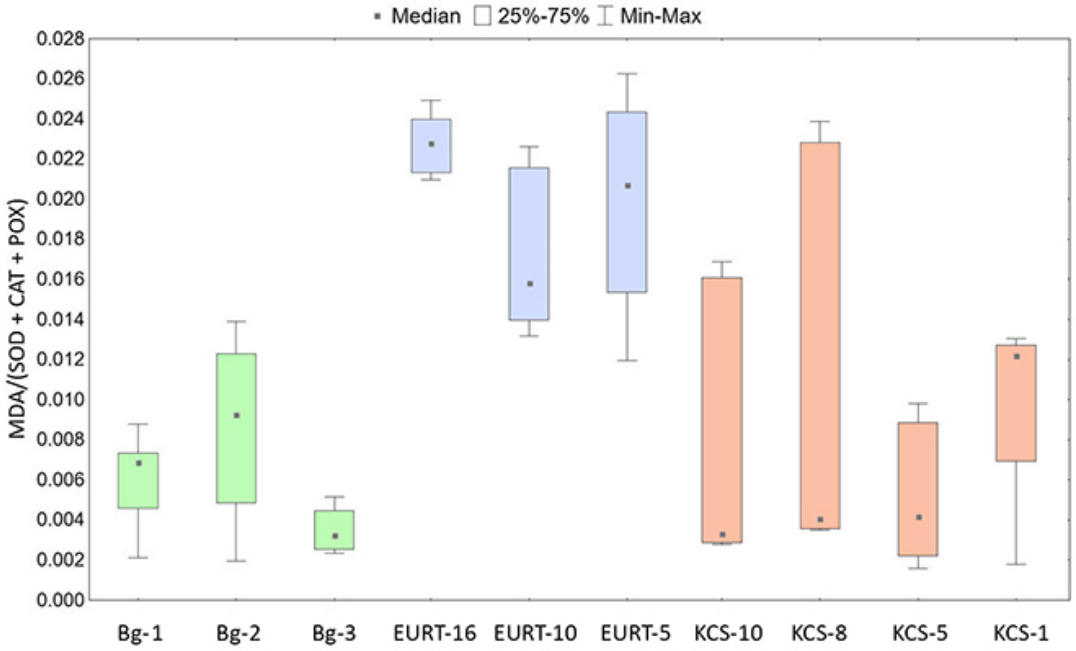

Fig. 3. Prooxidant/antioxidant index (MDA/(SOD + CAT + POX)) in *P. major* seedlings from the reference, KCS and EURT samples

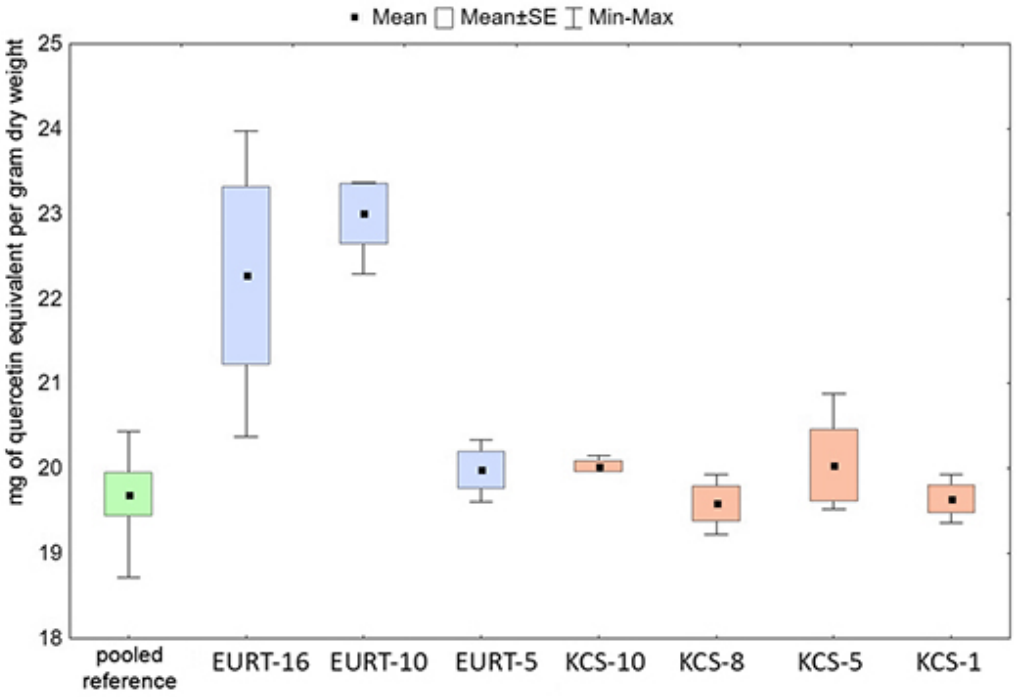

Fig. 4. Total low-molecular-weight antioxidants (mg of quercetin equivalent per gram dry weight) in *P. major* seedlings from different sites

PAI indices of the KCS samples and the reference ones (*U*-test, $n = 15–25$, $p = 0.45–0.52$).

Additionally, the analysis of the total LMWA (Fig. 4) in the seedlings of *P. major* was carried out, which showed no differences between the pooled samples from different sites ($H_{(1–3;6–12)} = 2.33–4.85$, $p = 0.067–0.183$). However, there was a significant increase in the total LMWA in the EURT-16 and EURT-10 samples, and, thus, in the pooled EURT sample, there was an increase in this parameter compared to the KCS and reference sites ($H_{(2;27)} = 9.85$, $p = 0.007$; Dunn test, $p = 0.013–0.038$).

Discussion

Analysis of the results obtained and their comparison with the literature data show that the functioning of the antioxidant system components and their relationship with the rates of lipid peroxidation processes are regulated differently depending on the type of stressor affecting them (Polesskaya, 2007; Shimalina et al., 2017, 2018; Słomka et al., 2008; Sharma et al., 2012; Morozova et al., 2016). The antioxidant system response is determined by the degree and duration of exposure to the stress (Polesskaya, 2007).

Differences in the mechanisms of ROS induction by ionizing radiation and heavy metals may be the cause of dissimilar reactions. The major process in the generation of reactive oxygen species by ionizing radiation is water radiolysis, which results in formation of hydrated electrons, superoxide radical, hydroperoxyl, and hydrogen peroxide (Grodzinsky, 1989; Esnault et al., 2010).

Heavy metals increase the generation of ROS through direct and indirect mechanisms, such as direct transfer of electrons in one-electron reactions; disturbance of metabolic pathways; displacement of irreplaceable cations from specific binding sites of enzymes and

inhibition of enzymatic activity due to affinity of heavy metals to SH-groups of enzymes; depletion of the pool of low-molecular-weight antioxidants (Shahid et al., 2014). Redox-active metals such as iron and copper can generate ROS directly by participating in biological redox reactions such as the Fenton and Haber-Weiss reactions (Shahid et al., 2014). The differences between plant responses to stress may be caused by the dissimilar chemical properties and biological activities of different ROS types, which are involved in processes occurring in various signaling systems (Apel, Hirt, 2004).

In the present study, MDA content and CAT and SOD activities were increased but POX activity was decreased in *P. major* from the EURT site. Another study (Volkova et al., 2017) also showed a decrease in peroxidase activity in *Pinus sylvestris* L. seedlings from the Chernobyl NPP zone and a high level of oxidative stress. However, no differences in the activities of superoxide dismutase and catalase were observed in those samples compared to the reference ones. In *Arabidopsis thaliana* (L.) Heynh. from the same zone, the activity of guaiacol peroxidase increased in the shoots, while activity of catalase and ascorbate peroxidase considerably decreased with increasing radiation load (Morozova et al., 2016). Thus, adaptive responses of the antioxidant system to the effect of ionizing radiation vary widely across species. The ability of plants to maintain prooxidant/antioxidant balance under toxic stress is also species specific, as it was shown for *Vicia cracca* L. and *Taraxacum officinale* Wigg. (Savinov et al., 2007), for *Melilotus albus* Merik. and *Trifolium medium* L. (Fazlieva et al., 2012).

One of the possible reasons for the diverse responses of plant antioxidant system to anthropogenic factors in nature may be the modifying effect of weather conditions (Pozolotina, Antonova, 2017). The ability of plants to tune their metabolism in a special mode, aimed at protecting them against excess ROS and associated oxidative stress, can be considered as a reaction developed during evolution that preserves the species potential for existence in adverse conditions.

Conclusion

The present study revealed the differences in the prooxidant/antioxidant balance of seed progeny of *P. major* from the sites with different types of anthropogenic contamination. Seedlings from the radioactively contaminated area exhibited a prooxidant shift: a higher content of malondialdehyde and an increased activity of superoxide dismutase (SOD) and catalase (CAT) compared to reference plants. In seedlings from the chemically contaminated area, the SOD and CAT activities were reduced compared to the reference sites, while the peroxidase activity was increased. Enhancement of antioxidant protection based on low-molecular-weight antioxidants was shown only in the East Ural Radioactive Trace samples. Results obtained in the present study indicate that dissimilarities in plant adaptive responses are caused by differences in the induction of reactive oxygen species under elevated ionizing radiation and under contamination by chemical toxicants.

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Activity of Cell Wall-Bound and Cytosolic Peroxidases under the Aftereffect of Copper Ions in *Nicotiana tabacum* Plants

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Abstract. The adaptation of plants to an excess of heavy metals in the environment and their recovery after elimination of the stressor is of interest in connection with the large-scale pollution of ecosystems and their remediation. This study is aimed at the aftereffect of copper ions (100 and 300 μM) in plants of *Nicotiana tabacum* L. The level of plant stress markers (concentration of hydrogen peroxide, activity of class III peroxidases – benzidine and guaiacol, their isoforms) during the recovery period after the removal of copper ions from the environment was evaluated in pretreatment by copper ions of different concentration and the use of control plants. During the recovery period, the concentration of hydrogen peroxide in plant organs (root, stem, and leaves) was high compared to the control. The responses of the roots and shoots under the aftereffect of the stressor were different. The activity of cytosolic guaiacol peroxidase and cell wall-bound peroxidases in root tissues increased according to the increase in H_2O_2. In plants pretreated with a lower copper concentration, the activity of cell wall-bound peroxidases in the stem and cytosolic and cell wall-bound benzidine peroxidases in leaves increased. In contrast, pretreatment with a high copper concentration led to a decrease in the activity of peroxidases during the period of plant recovery. Thus, plant organs differed in the content of H_2O_2 and the activity of class III peroxidases localized in different compartments (apoplast and cytosol) and in their ability to recover after the removal of the stressor.

Keywords: copper ions, *Nicotiana tabacum*, hydrogen peroxide, peroxidases, recovery.

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Активность ассоциированных с клеточной стенкой и цитозольных пероксидаз в условиях последействия ионов меди в растениях *Nicotiana tabacum*

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Аннотация. Адаптация растений к избытку тяжелых металлов в среде и их восстановление после элиминации стрессора представляют интерес в связи с масштабным загрязнением экосистем. Наше исследование направлено на изучение последействия ионов меди (100 и 300 μM) в растениях *Nicotiana tabacum* L. Оценивался уровень маркеров стресса растений (содержание пероксида водорода, активность пероксидаз III класса – бензидиновой и гваяколовой, их изоформы) в период их восстановления после удаления ионов меди из среды. Выявлено увеличение концентрации пероксида водорода в тканях корня, стебля и листьев. Реакции корня и побега в условиях последействия стрессора различались. Активность цитозольной гваяколовой пероксидазы и ассоциированных с клеточной стенкой пероксидаз в тканях корня повышалась на фоне увеличения содержания H_2O_2. Увеличение активности ассоциированных с клеточной стенкой пероксидаз в стебле, цитозольной и ассоциированной с клеточной стенкой бензидиновой пероксидазы в листьях наблюдали у растений, предобработанных более низкой концентрацией меди. Предварительная обработка высокой концентрацией меди, наоборот, приводила к снижению активности пероксидаз в период восстановления растений. Таким образом, органы растений различались по содержанию H_2O_2 и активности пероксидаз III класса, локализованных в разных компартментах (апопласт и цитозоль), и по способности восстанавливаться после снятия действия стрессора.

Ключевые слова: ионы меди, *Nicotiana tabacum*, пероксид водорода, пероксидазы, восстановление.

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Introduction

Copper, an essential trace element for plants, is necessary for enzyme activity, photosynthetic and respiratory electron carriers, chlorophyll biosynthesis, and nitrogen assimilation at low concentrations (Jouili et al., 2008; Ali et al., 2006). Excessive amounts of copper have a toxic effect: the efficiency of the electron transport chains in mitochondria and chloroplasts, organ biomass and size, and plant productivity decrease (Jouili et al., 2008; Lin et al., 2005). Many studies have been devoted to the study of plant responses to copper stress; however, there is much less information on plant recovery after the removal of stressors. Even after the copper ions were removed from the medium, they were transported from old organs to young organs and from cell walls to the protoplast, which can contribute to the development of oxidative processes in different organs even after the stressor is removed (Printz et al., 2016).

High concentrations of copper ions, like any stressor, provoke the formation of reactive oxygen species (ROS), including hydrogen peroxide. Antioxidant enzymes such as class III peroxidases (EC1.11.1.7), which include guaiacol (GPO) and benzidine (BPO) peroxidases, are involved in hydrogen peroxide metabolism. These antioxidant enzymes have an affinity for a wide range of oxidizable substrates and are localized in vacuoles and apoplasts, which distinguishes them from class I peroxidases (ascorbate peroxidase – EC1.11.1.11, glutathione peroxidase – EC1.11.1.9) located in chloroplasts, peroxisomes and cytoplasm (Veljovic Jovanovic et al., 2018). The activity of class III peroxidases specifically changes during plant development and depends on the type of plant tissue, the availability of substrates, and the strength and duration of the stress (Dragišić Maksimovic et al., 2008).

Apoplastic peroxidases use hydrogen peroxide and phenolic compounds as substrates, thereby maintaining the redox balance in plant cell walls and participating in the formation of monolignol radicals as monomers for lignin polymerization. Cytosolic isoforms of peroxidases, together with other antioxidant enzymes (superoxide dismutase, catalase, class I peroxidases), are involved in maintaining the ROS level in the protoplast, catabolism of auxins and anthocyanins, and porphyrin metabolism (Chamseddine et al., 2009).

Hydrogen peroxide is a more stable and long-lived molecule than other ROS (Schmitt et al., 2014). When transported across the plasma membrane, hydrogen peroxide can accumulate in the cell wall and cause a change in pH, which regulates the activity of apoplast enzymes through conformational rearrangements and phosphorylation (Jouili, El Ferjani, 2004). An increase in the hydrogen peroxide content at the stage of recovery in *Glycine max* L. plants pretreated with Cd^{2+} is considered one of the symptoms of toxicity (Holubek et al., 2020).

The optimum pH for GPO is 6.0–7.0, while the optimum pH for BPO is 5.0; therefore, these peroxidases are referred to as neutral and anionic isoforms, respectively. The role of class III peroxidases in the metabolism of hydrogen peroxide, which is formed under the action of an excess of heavy metals, has been shown in the plants *Helianthus annuus* L., *G. max*, and *Arabidopsis thaliana* L. (Jouili, El Ferjani, 2003; Lin et al., 2005).

The aim of our work was to study the aftereffect of different concentrations of copper ions on the activity of cell wall-bound and cytosolic peroxidases in *Nicotiana tabacum* L. in a long-term experiment.

Materials and methods

Plants of *N. tabacum*, cv. Petite Havana, line SR1 were cultivated on a preautoclaved substrate – a mixture of perlite: vermiculite in a 1:1 ratio, on Knop medium with the addition of 100 (variant 1) and 300 (variant 2) μM/L $CuSO_4$

and Knop medium as the control during the first 20 days after germination, followed by cultivation on Knop medium until reaching the age of 40 days. The plants were grown under conditions of a 16/8 photoperiod and a temperature of 23 °C.

The dry weight of plants was determined gravimetrically after fixing the material at 110 °C and drying it at 70 °C to constant weight. For each group, 30 plants were used.

The activity of enzymes guaiacol peroxidase (GPO, EC1.11.1.7) and benzidine peroxidase (BPO, EC1.11.1.7) and the content of hydrogen peroxide were determined spectrophotometrically on a Shimadzu UV-1800 (Shimadzu, Japan) in three biological and five analytical replicates. To obtain the supernatant, weighed samples of roots, stems, and leaves were homogenized in the cold in 0.05 M Tris-HCl buffer (pH 7.0) and centrifuged at 4 °C, and the extraction procedure was repeated twice. The resulting supernatant was used to measure the activity of cytosolic enzymes and the amount of hydrogen peroxide. To determine the activity of the cell wall-bound form of the enzyme, the precipitate after extraction of cytosolic enzymes was resuspended twice and centrifuged in 0.05 M Tris-HCl buffer (pH 7.0) supplemented with 1 M KCl (Jamet et al., 2006).

The BPO activity was determined spectrophotometrically as the rate of the oxidation reaction of benzidine in the presence of H_2O_2 at pH 5.0 and is expressed in relative units/mg protein × min (Goldfischer, Essner, 1969). The GPO activity was calculated as the rate of guaiacol oxidation using H_2O_2 as a substrate at pH 7.0 and was expressed in mM guaiacol/mg protein × min (Chance, Maehly, 1955). The protein content was determined according to Bradford (1976) using bovine serum albumin as a standard. Protein electrophoresis was performed under nondenaturing conditions in a 10 % polyacrylamide gel. Peroxidase isoforms were detected by a modified method (Lee et al., 2007): the gels were stained for 10 minutes in a reaction medium consisting of 0.2 % benzidine, 0.2 % guaiacol, and 2 % acetic acid. To remove excess substrate, gels were washed in 2 % acetic acid, then they were incubated for 3 minutes in 0.5 % hydrogen peroxide solution until clear bands appeared on an unstained background.

The amount of H_2O_2 was determined by the method based on the oxidation of xylenol orange chelates with iron(III) ions with hydrogen peroxide and was expressed in μM hydrogen peroxide/g dry weight (Bellincampi et al., 2000).

Statistical analysis was performed using STATISTICA 10 for Windows 10. Student's *t*-test was used for morphometric parameters, and the Mann-Whitney *U*-test was used for biochemical parameters. Correlations were estimated according to Spearman's nonparametric *R*-test.

Results

The level of oxidative stress in different organs of *N. tabacum* plants during the recovery period after exposure to different concentrations of Cu^{2+} was estimated by the content of H_2O_2 (Table 1). Compared to the control, the amount of hydrogen peroxide increased 2.1 and 4.5 times in the root, 1.4 and 1.6 times in the stem, and 1.4 and 2.3 times in the leaf in variants 1 and 2, respectively (the difference was statistically significant at $p = 0.03$).

To elucidate the role of peroxidase isoforms in the metabolism of hydrogen peroxide and adaptation of *N. tabacum* plants pretreated with high concentrations of copper ions, the activity of cytosolic (Fig. 1, A and B) and cell wall-bound isoforms of peroxidases (Fig. 1, C and D) were analyzed. Peroxidases were most active in the root tissues; in leaves, their activity in all variants of the experiment was lower. During the aftereffects of 100 and 300 μM Cu^{2+}, the activity of cytosolic GPO in roots increased significantly,

Table 1. Hydrogen peroxide content in tissues of *N. tabacum* plants after recovery from copper stress. The data are expressed as the mean with standard error.

Sample	H_2O_2, µM/g dry weight		
	Root	Stem	Leaf
Control (Knop medium)	0.71 ± 0.04	3.50 ± 0.07	8.43 ± 0.08
Variant 1 (100 µM Cu^{2+})	1.53 ± 0.13*	4.90 ± 0.19*	11.83 ± 0.36*
Variant 2 (300 µM Cu^{2+})	3.22 ± 0.02*a	5.56 ± 0.17*a	19.85 ±0.23*a

Note. *Statistically significant difference from the control ($p < 0.05$); a Statistically significant difference from variant 1 ($p < 0.05$) (Mann-Whitney *U*-test).

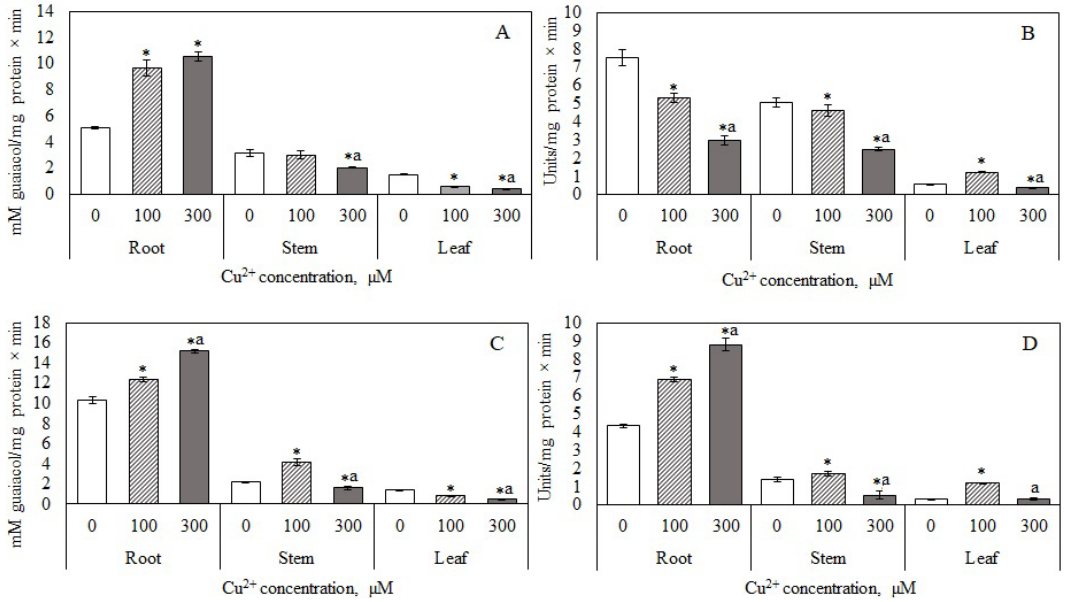

Fig. 1. Enzyme activities in *N. tabacum* organs after copper stress recovery: A – cytosolic guaiacol peroxidase; B – cytosolic benzidine peroxidase; C – cell wall-bound guaiacol peroxidase; D – cell wall-bound benzidine peroxidase. On the X-axis – copper ion concentration, µM; on the Y-axis – enzyme activity. The data are expressed as the mean with standard error. *Statistically significant difference from the control ($p < 0.05$); a statistically significant difference from variant 1 ($p < 0.05$) (Mann-Whitney *U*-test)

by 90 and 106 %, respectively (Fig. 1, A). In contrast, the activity of cytosolic BPO decreased (Fig. 1, B) by 30 and 60 % relative to the control in variants 1 and 2, respectively (the difference was significant, $p = 0.01$).

In the stems of plants pretreated with 100 µM Cu^{2+}, the activity of cytosolic GPO and BPO did not change. In the case of pretreatment with 300 µM Cu^{2+}, their activity decreased by 36 and 51 %, respectively (the difference was statistically significant, $p = 0.03$).

At the stage of recovery, a decrease in the activity of cytosolic GPO observed in leaves was 58 and 73 % compared to the control in variants 1 and 2, respectively. The activity of cytosolic BPO upon pretreatment with 100 µM Cu^{2+} significantly increased 2.1 times; however, the activity decreased by 37 % in the case of pretreatment with 300 µM Cu^{2+}.

The activity of cell wall-bound GPO (Fig. 2, C) and BPO (Fig. 2, D) in roots increased radically during the aftereffect period of high

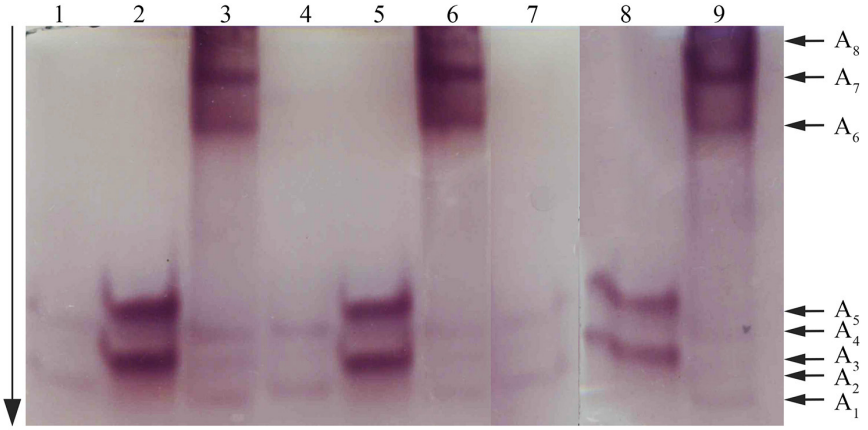

Fig. 2. Isoforms of cytosolic peroxidase in *N. tabacum* organs after copper stress recovery: the arrow indicates the direction of current flow; 1 – leaf, control (0 μM Cu^{2+}); 2 – stem, control (0 μM Cu^{2+}); 3 – root, control (0 μM Cu^{2+}); 4 – leaf, variant 1 (100 μM Cu^{2+}); 5 – stem, variant 1 (100 μM Cu^{2+}); 6 – root, variant 1 (100 μM Cu^{2+}); 7 – leaf, variant 2 (300 μM Cu^{2+}); 8 – stem, variant 2 (300 μM Cu^{2+}); 9 – root, variant 2 (300 μM Cu^{2+})

concentrations of copper ions. In variant 1 in the stem, the activity of GPO increased by 88 % and BPO by 22 % compared to the control (the difference was statistically significant, $p = 0.03$). In contrast, in variant 2, a significant decrease in the activity of GPO and BPO was noted as 26 and 62 %, respectively (at $p = 0.03$). In leaf tissues, the activity of peroxidases changed in a different way. The activity of cell wall-bound GPO was comparable to the activity in the control plants in the case of pretreatment with 100 μM Cu^{2+} but significantly decreased in the case of 300 μM Cu^{2+}. The BPO activity in variant 1 increased by 3.5 times (the difference was statistically significant, $p = 0.03$); in variant 2, the change in activity was not significantly different.

The activity of cytosolic and cell wall-bound isoforms of peroxidases in the root tissues was higher than the activity in the stem and leaves (Fig. 1). At the stage of recovery after the copper ions were removed, an increase in the activity of cytosolic GPO and cell wall-bound GPO and BPO was found. However, the activity of cytosolic BPO (Fig. 1, B) decreased in roots and stems, different from the general trend of the effect of pretreatment with copper ions on peroxidase activity in different parts of plants. To explain this fact, protein electrophoresis of cytosolic peroxidases isolated from root, stem, and leaf tissues was performed (Fig. 2). Isoforms A_2 and A_4 were common in root, stem, and leaf tissues; their activity decreased under stress conditions. Four isoforms of peroxidases were identified in the stem tissues: A_2-A_5. In the case of plant pretreatment with 300 μM Cu^{2+}, a decrease in enzyme activity was noted. Three specific isoforms, A_6-A_8, were found in root tissues. The activity of these three isoforms remained high in all variants of the experiment. We assume that the total BPO activity decreased in root tissues due to a decrease in the activity of A_2 and A_4 isoforms.

Peroxidase isoforms in roots and shoots were reported to differ in sensitivity to ROS (Dragišić Maksimovic et al., 2008). In our study, at the stage of plant recovery after copper stress, an increase in the content of hydrogen peroxide was accompanied by an increase in the activity of cell wall-bound BPOs (Spearman's correlation coefficient r = +0.85), cell wall-bound (r = +0.76) and cytosolic GPO (r = +0.77) in the root and a decrease in the activity of cytosolic BPO

(r = –0.84). In the stem, the activity of cytosolic peroxidases decreased with an increase in hydrogen peroxide amount (r = –0.66). In leaves, no statistically significant correlations were found between H_2O_2 production and enzyme activity. The changes in enzyme activity indicate that the plants did not eliminate the symptoms of copper ion toxicity and were still stressed even after the treatment was remote for 20 days. The continuation of stress was also confirmed by the morphometric analysis data. At the stage of recovery after Cu^{2+} ions were removed from the media, *N. tabacum* plants revealed changes in their growth. The biomass was 0.45 ± 0.04 g in the control, 0.50 ± 0.04 g in variant 1 (100 μM Cu^{2+}), and 0.34 ± 0.02 g in variant 2 (300 μM Cu^{2+}). The plants in variant 1 were comparable to the control group, and in variant 2, a statistically significant decrease in dry weight by 24 % relative to the control was noted (at $p = 0.03$).

Discussion

The level of oxidative stress in different organs can be characterized by the amount of hydrogen peroxide. The data obtained revealed H_2O_2 accumulation in different organs of *N. tabacum* pretreated with copper ions, as Cu^{2+} is a stimulator of ROS production in the Fenton and Haber-Weiss reactions (Elleuch et al., 2013). Most likely, the high level of hydrogen peroxide in plants after a twenty-day recovery period was caused by the redistribution of copper ions between organs and translocation from the cell walls to the protoplast (Printz et al., 2016; Marques et al., 2018). We assumed that high concentrations of H_2O_2 in root tissues stimulated the activity of cytosolic and cell wall-bound GPO and cell wall-bound BPO, which could lead to increased lignification of cell walls, binding of Cu^{2+} by carboxyl, hydroxyl, and other groups of lignin, and a decrease in the translocation of metal ions into the shoot (Bouazizi et al., 2011).

The activity of peroxidases is considered to be a marker of plant resistance to stress factors. The induction of peroxidase activity was shown to be most typical for plants sensitive to an excess of Cu^{2+} in the medium, while resistant forms of plants are characterized by low peroxidase activity (Bouazizi et al., 2007). Copper ions induced the activity of anionic peroxidases and lignin biosynthesis in the cell walls of the root in *H. annuus* and *G. max* (Jouili, El Ferjani, 2003; Lin et al., 2005). In response to 50 μM Cu^{2+}, the activity of cytosolic and apoplastic GPO in the leaves and stems of *H. annuus* increased (Jouili, El Ferjani, 2004).

The different trends in changes in cytosolic and cell wall-bound BPO activity in root tissues under stress conditions could be a consequence of local changes in the pH level in the cell walls and cytosol during the recovery period after the removal of the stressor. In *Phaseolus vulgaris* L., after treatment with 50 and 75 μM Cu^{2+}, the activity of apoplastic coniferyl alcohol peroxidase in root tissues increased but decreased in the cytosol (Bouazizi et al., 2011). The authors suggest that under copper-induced stress, the enzyme was transported from the protoplast to the apoplast, which led to a decrease in the activity of the cytosolic form. An increase in the activity of class III peroxidase isoforms associated with the cell wall may indicate the role of lignification as an adaptive response to an excess of copper ions in the medium (Bouazizi et al., 2011). Most likely, similar mechanisms contributed to the induction of the activity of BPO apoplastic isoforms.

Since GPO and BPO have different pH optima, localize in tissues and differ in affinity to substrates, changes in their activity in different organs and tissues can be a specific reaction in the recovery period after stress. We assume that in roots, copper ions were predominantly deposited in cell walls of the cortex, partially in the vacuoles; in shoots, the content of copper ions

was higher in the cytoplasm than in the cell walls. The deposition of heavy metals in the vacuole of the parenchymal cells of the cortex and stele in shoots was shown earlier (Printz et al., 2016; Marques et al., 2018). The data obtained showed that physiological and biochemical processes were restored in plants pretreated with 100 μM Cu^{2+}, which can be proven by the absence of significant changes in the activity of cytosolic peroxidases compared to the control plants. A decrease in the activity of cell wall-bound and cytosolic enzymes at 300 μM Cu^{2+} may be a consequence of deeper damage to the structure and synthesis of proteins and incomplete plant recovery at 20 days (Bouazizi et al., 2007).

The level of hydrogen peroxide in control and prestressed plants was higher in the leaf tissues and lower in the roots, which may be due to metabolic processes, especially the formation of ROS in light reactions of photosynthesis and in photorespiration. Since there were no correlations between the activity of class III peroxidases and the level of hydrogen peroxide in leaves, we assumed that in leaves, other antioxidant enzymes were involved in maintaining the redox balance, such as class I peroxidases and catalase (Chamseddine et al., 2009; Veljovic Jovanovic et al., 2018).

The observed changes, in particular, the decrease in plant biomass in the case of pretreatment with 300 μM Cu^{2+}, indicate that there was not complete recovery of *N. tabacum* plants within 20 days after the removal of the excess copper ions from the media, and the plants did not eliminate the symptoms of toxicity. A decrease in organ biomass is a nonspecific reaction to heavy metal stress. For example, *Withania somnifera* L. under 200 μM Cu^{2+} was characterized by a decrease in the total mass and mass of individual organs (Khatun et al., 2008). The decrease in dry mass was also described for *P. vulgaris* leaves under treatment with 160 μM Cu^{2+} (Cook et al., 1997). In *Glycine max* L. pretreated with higher concentrations of Cd^{2+}, the restoration of shoot and leaf growth and a reduction in root length were described (Holubek et al., 2020). Our data also showed a decrease in the mass of *N. tabacum* plants. We suppose that this effect was associated with the overproduction of ROS and the redistribution of plant resources for the synthesis of enzymes and low molecular weight antioxidants.

Conclusions

The data obtained revealed that in tobacco plants pretreated with copper ions by 100 or 300 μM $CuSO_4$, the responses of roots, stems, and leaves during the recovery period after stress were different. The aftereffect of excess copper ions led to an increase in the content of hydrogen peroxide in roots and shoots, which indicates the sensitivity of *N. tabacum* to this stressor and incomplete plant recovery in the poststress period. In the case of plant pretreatment with 100 μM Cu^{2+}, a specific increase in the activity of cell wall-bound GPO and BPO was observed in the stem, and an increase in the activity of BPO in apoplast and cytosol was observed in the leaves. An increase in cytosolic GPO activity in roots, caused by an increase in H_2O_2, led to a decrease in the level of oxidative stress in the cytosol. The increase in cell wall-bound peroxidase activity could lead to the higher formation of monolignol radicals, which, in turn, could cause additional lignification and a partial restriction of copper ion transportation into the shoot. Thus, an increase in the activity of class III peroxidases promoted a decrease in oxidative processes in cells and restoration of plant growth. We assume that plants pretreated with 300 μM Cu^{2+} require a recovery period longer than 20 days, since they retained the markers of copper ion toxicity after the stressor was removed.

The data obtained indicate the heterogeneity of tissues and organs in response to oxidative stress caused by the action of copper ions in the culture medium. The results show that copper had a long-lasting aftereffect. The accumulation of H_2O_2 and the activity of peroxidases depend on the strength of the stress and their tissue localization.

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The Effect of UV-B Radiation on the Antioxidant System in the *Peltigera aphthosa* and *Peltigera rufescens* Lichens

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Abstract. Ultraviolet (UV) radiation is the short wavelength region of the solar spectrum. The high-energy photons of UV-B (280–315 nm) are potentially dangerous for all living organisms. The effect of UV-B radiation on lichens has not been studied sufficiently. We conducted a comparative study of the effects of the long-term (10 d) exposure to the environmentally realistic dose of UV-B radiation on the accumulation of lipid peroxidation products (TBARS), H_2O_2 content, superoxide dismutase (SOD) activity, and respiration rate in *Peltigera aphthosa* from the forest community and *Peltigera rufescens* from the open spaces of floodplain meadow. The H_2O_2 content and the SOD activity were found to increase in the thalli of *P. rufescens*. The TBARS content in the UV-B treated thalli of *P. rufescens* did not differ from the control thalli and was 2.5 times higher than in *P. aphthosa*. In *P. aphthosa* thalli, SOD activity did not change after UV-B exposure, and TBARS content increased by 33 % with an increase in the total UV-B dose. Both lichens exhibited an increase in the alternative respiratory pathway (AP) activity and a decrease in the ratio of the main (cytochrome) pathway to the energy low efficient AP. The AP involvement was more pronounced in *P. aphthosa*. The results of our study indicate the species-specific response in lichens and differences in their resistance to oxidative stress, which were due to adaptation to the light conditions in the typical habitats of these species.

Keywords: *Peltigera aphthosa*, *Peltigera rufescens*, lichens, UV-B radiation, lipid peroxidation, hydrogen peroxide, superoxide dismutase, alternative respiratory pathway, resistance.

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Влияние УФ-В-радиации на антиоксидантную систему лишайников *Peltigera aphthosa* и *Peltigera rufescens*

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Аннотация. Ультрафиолетовая (УФ) радиация относится к коротковолновой части солнечного спектра. Высокоэнергетические фотоны УФ-В-излучения (280–315 нм) потенциально опасны для всех живых клеток. Реакция лишайников на УФ-В-излучение исследована недостаточно. Мы провели сравнительное изучение влияния длительного действия экологически обоснованной дозы УФ-В-радиации на накопление продуктов перекисного окисления липидов (ПОЛ), содержание H_2O_2, активность супероксиддисмутазы (СОД) и дыхание талломов *Peltigera aphthosa* из лесного сообщества и *Peltigera rufescens*, обитающей на хорошо инсолируемых участках пойменного луга. Выявили повышение содержания H_2O_2 и активности СОД в талломах *P. rufescens*, экспонированных к УФ-В. Содержание продуктов ПОЛ в импактных талломах *P. rufescens* не отличалось от контроля и было в 2,5 раза выше по сравнению с *P. aphthosa*. Уровень активности СОД в талломах *P. aphthosa* не изменялся, но содержание продуктов ПОЛ возрастало на 33 % с увеличением суммарной дозы УФ-В. У обоих видов лишайников отмечали повышение активности альтернативного пути (АП) дыхания, что приводило к изменению соотношения основного (цитохромного) и энергетически малоэффективного АП. Вовлечение АП было сильнее выражено у *P. aphthosa*. Результаты исследования свидетельствуют о видовой специфичности реакции лишайников на воздействие УФ-В-излучения, а также указывают на различия в их устойчивости к окислительному стрессу, обусловленные приуроченностью видов к местообитаниям с разным режимом освещенности.

Ключевые слова: *Peltigera aphthosa*, *Peltigera rufescens*, лишайники, УФ-В-радиация, перекисное окисление липидов, пероксид водорода, супероксиддисмутаза, альтернативный путь дыхания, устойчивость.

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Introduction

Lichens are a stable self-regulating association of heterotrophic mycobionts with photoautotrophic green algae and/or cyanoprokaryotes. The mycobiont accounts for over 90 % of the thallus biomass and most of the respiration (Palmqvist et al., 2008). The symbiotic nature and poikilohydric properties of lichens ensure that these organisms have high resistance to adverse environmental stresses (Kranner et al., 2005). Due to this, lichens occupy various stressful terrestrial habitats, including deserts, highlands, Arctic and Antarctic regions, and high stress microhabitats in less stressful habitats.

Of the sun ultraviolet (UV) radiation spectrum, only near-UV-A radiation (315–400 nm) and a small portion of UV-B photons (280–315 nm) reach the Earth's surface. The high-energy UV-B photons can damage biologically important macromolecules and induce oxidative stress in the cells of living organisms (Frohnmeyer, Staiger, 2003). Adaptive (architecture modification, synthesis of a range of secondary metabolites) and nonspecific harmful (damage to DNA, proteins and membranes, inhibition of photosynthesis and growth, accumulation of reactive oxygen species (ROS)) effects of UV-B radiation on plants have been well-documented (Caldwell et al., 1995, 2003; Jenkins, 2009). Fewer data are available on the effects of UV-B on lichens. High doses of UV-B radiation are known to induce programmed cell death (Ünal, Uyanikgil, 2011) and suppress the growth of lichen thalli (Chowdhury et al., 2017). Different secondary lichen compounds synthesized by the mycobiont play an important role in protecting the lichen photobiont against excessive visible light and UV radiation (Nguyen et al., 2013). UV-B induces the synthesis of parietin and melanins in the thalli of some lichen species (Solhaug et al., 2003; Nybakken et al., 2004; Solhaug, Gauslaa, 2012; Mafole et al., 2019). These compounds are synthesized in the thallus upper cortex cells and absorb or reflect UV (A+B) rays, thereby shielding the underlying layers of cells.

Most lichen species in the Komi Republic grow in forest communities. The boreal species often prefer shaded and moist habitats. In the forest, lichens are rarely exposed to the direct sunlight. Such lichens can serve as a convenient model for studying the adverse effects of UV-B radiation. For comparison, lichens from the open habitats, which are exposed constantly to UV radiation, are of great interest for studying protective mechanisms against the UV-B impact. We hypothesized that these species differ considerably in their antioxidant system activity and protective reactions to oxidative stress. To test this idea, we conducted a comparative study of the UV-B effects on the activity of the key antioxidant enzyme – superoxide dismutase (SOD), the H_2O_2 content, and the level of lipid peroxidation in *Peltigera aphthosa* (L.) Willd. and *Peltigera rufescens* (Weiss) Humb. These lichen species grow in different types of habitats. The effect of UV-B on the respiration rate and the ratio of the cytochrome pathway to the alternative respiratory pathway was studied also to estimate changes in lichen metabolism.

Materials and methods

Lichens

P. aphthosa is a foliose lichen with a circumpolar distribution. It is found in the Arctic, boreal, and temperate zones. The lichen grows on moss, soil, and plant debris in shaded and moist sites (Thomson, 1984). The main photobiont of *P. aphthosa* is the green algae of the *Pseudococcomyxa* genus. The cephalodia on the thallus surface contain cyanoprokaryotes of the genus *Nostoc*.

P. rufescens is a foliose cyanolichen. Its thallus contains cyanobacteria of the *Nostoc* genus. This species has a polyzonal distribution and grows in the temperate and boreal latitudes of the Northern hemisphere and in South America and Australia, in fully sunlit habitats, on the open sites in the fields and roadsides (Brodo et al., 2001).

Sampling sites

The study was conducted in the summer of 2018. *P. aphthosa* thalli were collected in a pine-dominated forest mixed with spruce and deciduous trees, in shaded and moist places. The thalli of *P. rufescens* were collected in open, well-lit areas of meadows adjacent to the river Vym floodplain. The meadow soil was a well-drained sod-layered sandy loam.

Lichen samples were collected at midday, during clear sunny weather. Simultaneously with sampling, the photosynthetic active radiation (PAR) intensity, air temperature, and relative humidity were measured using a portable weather station (Data Logger LI-1400, U.S.A.). The intensity of near-UV (UV-A and UV-B) radiation was determined using a UV radiometer (TKA-PKM 12, Russia). The thalli were cleaned to remove substrate residues and transported to the laboratory, where air-dry thalli were stored in the dark at 4 °C.

Experiment design

Before starting the UV radiation treatment, the thalli were moistened and kept for 3 d under fluorescent lamps (Philips TL-D Aquarelle) at 80 μmol m^{-2} s^{-1} (10/14 h photoperiod, 25 °C). After that, the thalli were exposed to UV from lamps (LER40, Russia) for 10 d (2 h d^{-1}). The peak emission spectrum of the lamp was 315 nm and the UV-B irradiation intensity was 2 W m^{-2}. Consequently, the thalli of lichens received about 14 kJ d^{-1} of the UV-B light. That was an environmentally realistic dose, corresponding to the daily dose of UV-B radiation that reaches open ground in this region on a sunny summer day.

The effect of UV-B treatment on thalli was analyzed after 1, 3, and 10 d, which corresponded to the total dose of UV-B radiation of 14, 43, and 144 kJ, respectively. The control thalli were not treated with UV radiation.

Biochemical analyses

The level of lipid peroxidation was estimated according to the method of Heath and Packer (1968) by assaying the thiobarbituric acid reactive substances (TBARS). The concentration of H_2O_2 was measured using the method of Bellincampi et al. (2000). The activity of SOD was determined by its ability to inhibit the photochemical reduction of nitroblue tetrazolium (Beauchamp, Fridovich, 1971). The protein content was determined according to the method of Bradford (1976) with bovine serum albumin taken as the standard.

The respiration rate (V_{total}) was determined by performing the polarographic measurement of O_2 uptake using the Oxytherm System Clark electrode (Hansatech Instruments, U.K.). The marginal regions of the lichen thalli were used. The cytochrome (V_{cyt}) and alternative respiratory capacities (V_{alt}) and their ratio were estimated using specific inhibitors (Bahr, Bonner, 1973). The inhibitors – 6 mM benzhydroxamic acid (Lancaster, U.K.) and 2 mM KCN (Sigma Aldrich,

St. Louis, MO, U.S.A.) – were added successively after measuring the total O_2 uptake rate.

Statistical analysis

The results are presented as means with standard errors (SE) of n (n = 3–8) for the control and for samples exposed to each UV-B dose. After checking for normal distribution of variables (Shapiro-Wilk's test), data were analyzed using one-way ANOVA followed by post hoc multiple range testing (Duncan's test, $P < 0.05$). Tests were conducted using Statistica 10.0 software (StatSoft. Inc., U.S.A.).

Results

Microclimatic conditions at the sample sites

The habitats of the lichens studied in the present work differed in the amount and quality of light (Table 1). In sunny weather, *P. rufescens* thalli in the meadow were exposed to PAR intensity that was 5 times higher and UV (A+B) intensity that was one order of magnitude higher compared to *P. aphthosa* in the forest. In the meadow, the air warmed up to an average of 31 °C, while in the forest, the air temperature was 10 °C lower.

Level of lipid peroxidation

The widely used thiobarbituric acid-reactive-substances (TBARS) assay measures free malondialdehyde (MDA). MDA is largely the product of peroxidation of fatty acids with more than two double bonds. We found that the content of TBARS in the control thalli of *P. rufescens* was 3 times greater than in *P. aphthosa* (Table 2) indicating that UV-B exposure increased fatty acid peroxidation in a species-specific way. After 3 d, the TBARS content in *P. aphthosa* increased by 33 % compared with the control and did not change after that, until the end of the

Table 1. Spot measurement of the microhabitat conditions in the lichen sampling sites (means ± SE, n = 6–8)

Parameter	Meadow	Forest
PAR, µmol photons (m^{-2} s^{-1})	1461 ± 42	311 ± 18
Intensity of UV (A+B) radiation, W m^{-2}	26.9 ± 0.5	1.4 ± 0.1
T_{air}, °C	31.1 ± 0.3	21.2 ± 0.4
RH, %	35.7 ± 2.3	40.3 ± 1.4

Table 2. Lipid peroxidation level and H_2O_2 content in the thalli exposed to UV-B radiation

Total dose of UV-B, kJ (day)	*Peltigera aphthosa*		*Peltigera rufescens*	
	TBARS, nmol g^{-1} DW	H_2O_2, µmol g^{-1} DW	TBARS, nmol g^{-1} DW	H_2O_2, µmol g^{-1} DW
Control	150.0 ± 3.2^{a}	41.4 ± 3.0^{a}	464.7 ± 9.2^{a}	36.0 ± 1.4^{c}
14 (1)	143.7 ± 3.3^{a}	40.5 ± 1.1^{a}	487.5 ± 9.3^{a}	42.3 ± 1.8^{a}
43 (3)	196.0 ± 7.0^{b}	50.7 ± 1.3^{c}	540.6 ± 21.8^{b}	48.5 ± 1.5^{b}
144 (10)	196.2 ± 6.2^{b}	30.3 ±0.5^{b}	473.9 ± 3.9^{a}	43.7 ± 1.8^{ab}

Lipid peroxidation products (TBARS) and H_2O_2 content are presented as means ± SE (n = 3–4). Significant differences between values depending on total UV-B dose are indicated by different superscript letters (one-way ANOVA, Duncan test, $P < 0.05$). DW – dry weight.

experiment. By contrast, in *P. rufescens*, after 3 d, the TBARS content was only 16 % higher than in the control but did not differ from the control after 10 d.

Hydrogen peroxide content

The lichen species did not significantly differ in the hydrogen peroxide levels (Table 2). The maximum accumulation of H_2O_2 (48–50 μmol g^{-1} DW) in the thalli of both lichen species occurred after 3 d, and it was 20–25 % higher than in the control. The H_2O_2 content decreased by 40 % in *P. aphthosa* and did not change in *P. rufescens* after 10 d of UV-B exposure.

Superoxide dismutase activity

The control thalli of *P. aphthosa* and *P. rufescens* showed similar levels of SOD activity (Table 3). The UV-B treatment did not affect the SOD activity in *P. aphthosa* thalli. We observed a significant increase in the SOD activity in *P. rufescens* thalli after 3 and 10 days of the UV-B exposure. After 10 d of UV-B treatment, the enzyme activity in the thalli of *P. rufescens* was 35 % higher than in the control.

Respiration

Total O_2 uptake rate in the control and UV-treated thalli of *P. rufescens* was more than 1.5 times higher than in *P. aphthosa* (Table 4). UV-B radiation significantly affected the total O_2 uptake rate and the proportions of the activities of the respiratory pathways. A decrease in total O_2 uptake rate and in cytochrome pathway (CP) activity was observed immediately after 1 d in both species. The alternative pathway (AP) capacity increased and the CP/AP ratio decreased by 2.5 times compared to the control samples. After 3 d, total O_2 uptake increased, but the CP/AP

Table 3. Superoxide dismutase activity (units mg^{-1} protein) in the thalli exposed to UV-B radiation

Total dose of UV-B, kJ (day)	*Peltigera aphthosa*	*Peltigera rufescens*
Control	12.2 ± 0.3^{a}	11.1 ± 0.3^{a}
14 (1)	11.9 ± 0.2^{a}	11.0 ± 0.2^{a}
43 (3)	12.0 ± 0.2^{a}	12.8 ± 0.3^{b}
144 (10)	11.8 ± 0.1^{a}	17.3 ± 0.5^{c}

Enzyme activity is presented as means ± SE (n = 3–4). Other designations as in Table 2.

Table 4. Influence of UV-B radiation on the total respiration rate and the activities of the cytochrome and alternative respiratory pathways in lichen thalli (nmol O_2 g^{-1} DW min^{-1})

Total dose of UV-B, kJ (day)	*Peltigera aphthosa*		*Peltigera rufescens*	
	V_{total}	V_{cyt}/V_{alt}	V_{total}	V_{cyt}/V_{alt}
Control	939 ± 39^{b}	3.6 ± 0.7^{b}	1306 ± 56^{a}	3.3 ± 0.5^{b}
14 (1)	740 ± 34^{a}	1.3 ± 0.2^{ab}	1153 ± 7^{ab}	1.5 ± 0.2^{a}
43 (3)	1042 ± 39^{b}	1.3 ± 0.3^{ab}	1343 ± 51^{a}	1.4 ± 0.1^{a}
144 (10)	660 ± 47^{a}	0.9 ± 0.1^{a}	1069 ± 73^{b}	1.3 ± 0.1^{a}

O_2 uptake rate (V_{total}), cytochrome and alternative respiratory pathway activities ratio (V_{cyt}/V_{alt}) are presented as means ± SE (n = 5–15). Other designations as in Table 2.

ratio did not change. After 10 d, total O_2 uptake rate was 35 % lower than after 3 d and 30 % lower than in the control thalli of *P. aphthosa*. The decrease in *P. rufescens* respiration rate was less pronounced. The value of CP/AP ratio remained low in both species.

In the control thalli of both species, the proportion of CP accounted for more than 60 % of the total respiration rate (Figure). The contribution of AP to total O_2 uptake was only 20 %. Increased involvement of the low energy effective alternative pathway and a decrease in the main (cytochrome) pathway contribution to the total respiration were observed in UV-B treated thalli. The CP contribution decreased on average by 1.4 times and the AP contribution doubled after 10 d of exposure to UV-B radiation. The contributions of AP to the total respiration of *P. aphthosa* and *P. rufescens* thalli were 44 % and 35 %, respectively.

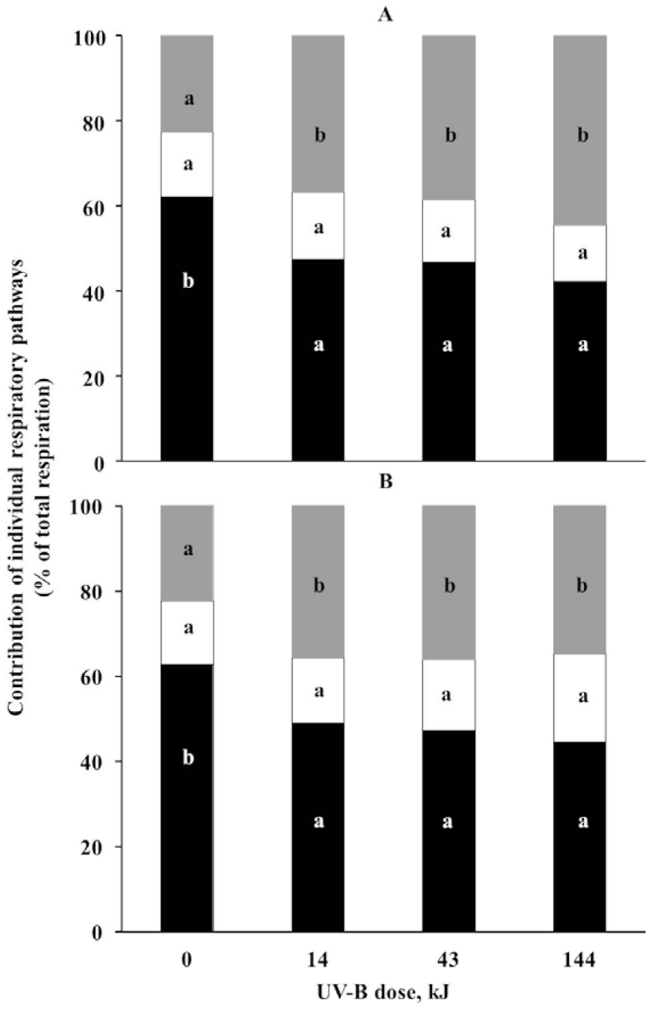

Figure. Effect of UV-B radiation on the relative contribution of each respiratory pathway to total respiration in the lichens A) *Peltigera aphthosa* and B) *Peltigera rufescens*. The contributions of the cytochrome (black) and alternative pathways (grey) and residual respiration (white) to total respiration are shown. Different letters in each column indicate significant differences between the control and different UV-B doses (one-way ANOVA, Duncan test, $P < 0.05$). 0 – Control samples without UV-B exposure. 14, 43, 144 – The total dose of UV-B radiation received by thalli on the first, third and tenth days of exposure, respectively

Discussion

The aim of this work was to obtain information on the effects of UV-B radiation on the metabolism of *P. aphthosa* and *P. rufescens*. In nature, the lichen thalli grow at different levels of insolation and temperature (Table 1). High insolation, especially the high-energy short-wavelength photons, increases the generation of ROS, which causes oxidative stress in living cells. The effect of high-energy photons on lichens depends on their state. It is believed that ROS can attack membrane lipids, proteins, and other biologically significant molecules in the wet lichens. The dry lichens are inactive and resistant to extreme conditions. They retained their vital functions even after exposure to open space conditions (De Vera et al., 2008; Sánchez et al., 2014). However, *Cladonia arbuscula* ssp. *mitis* (Sandst.) Ruoss was sensitive to UV-B irradiation in the air-dried state and was not able to completely repair the DNA damage (Buffoni Hall et al., 2003). Furthermore, temperature and light intensity played a role in the capacity of the lichen to self-repair the damage.

Our results show that the two lichens studied here exhibited different physiological responses to an environmentally realistic dose of UV-B. We found that *P. rufescens* thalli had a higher TBARS content but accumulated considerably smaller amounts of lipid peroxidation products under UV-B treatment compared to *P. aphthosa* (Table 2). This is most likely due to the adaptation of *P. rufescens* to brighter light and higher temperature conditions in its microhabitat. However, interestingly, an increase in H_2O_2 content in *P. rufescens* thalli was observed shortly after exposure to UV-B light while in *P. aphthosa*, the H_2O_2 content increased significantly only after 3 d, when the total UV-B dose was around 40 kJ.

The control thalli of both species did not differ in SOD activity. SOD is highly efficient in catalytic removal of the superoxide radical ($O_2^{\bullet-}$). SOD catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2. H_2O_2 is a relatively stable compound, which regulates many cell processes. We did not find a direct relationship between changes in the SOD activity and the content of H_2O_2 following UV-B exposure. The UV-B treatment had no effect on SOD activity in *P. aphthosa* thalli. Rehydration of desiccated lichens did not cause any response or decrease in SOD activity (Weissman et al., 2005). A «burst-like» formation of ROS and, in particular, the superoxide radical, was observed in lichens after a long desiccation period (Beckett et al., 2003). Probably, any upregulation of SOD during rehydration was suppressed due to inactivation by high ROS concentrations in thalli. The increase in TBARS content in *P. aphthosa* thalli was probably a result of the accumulation of ROS. Lichens can contain from six to ten Fe-, Cu/Zn-, and Mn-SOD isoforms (Schlee et al., 1995; Weissman et al., 2005). UV could have different effects on the activity of different SOD isoforms, which resulted in the absence of the pronounced effect on the total SOD activity. A significant increase in the SOD activity in *P. rufescens* thalli was observed after 3 d of the treatment. SOD activity was maximal after 10 d of exposure, when the total UV-B dose reached 144 kJ, indicating that UV-B increased the capacity of this species to dismutate $O_2^{\bullet-}$ into more stable H_2O_2. A more rapid conversion of $O_2^{\bullet-}$ to H_2O_2 can contribute to a more successful defense of cells from the most active ROS. At the same time, an increase in the H_2O_2 content also contributes to the development of oxidative stress, and future work needs to focus on the effect of UV-B on the enzymes, such as catalase, that metabolize H_2O_2.

Respiration is a crucial process that provides all living organisms with energy and metabolites for growth and cellular maintenance. We found that UV-B treatment had a significant effect on the total respiration rate and CP/AP

ratio in the thalli of both lichen species studied here (Table 4). The activity and proportion of AP in total respiration increased, whereas the contribution of CP decreased (Figure), suggesting that the UV-B treatment activated the energy dissipation processes. The effect of UV-B on the respiratory pathway ratio was more pronounced in *P. aphthosa* than in *P. rufescens*. We noted the same reaction to UV-B in *Cladonia stellaris* (Opiz) Pouzar & Vezda (Shelyakin et al., 2018). The change in the ratio of the respiratory pathways in UV-B treated lichens is probably a common event. The activation of AP may prevent an excessive reduction in the mitochondrial electron transport chain and the development of oxidative stress in cells. The role of the alternative respiratory pathway as a component of the ROS-scavenging system in lichens has already been discussed by other researchers (Beckett et al., 2008). Since the fungal component accounts for more than 90 % of the lichen thalli, we assume that the mycobiont is the main cause of the changes in the ratios of the respiratory pathways found here. The question of the contribution of mycobiont and different types of photobiont to the total respiration, peroxide accumulation, and the activity of antioxidant enzymes in lichens needs further investigation.

Conclusion

The results demonstrate that the response to UV-B radiation of the antioxidant system and respiration of lichens is species-specific. *P. rufescens*, growing in the open habitat, accumulates greater amounts of products of lipid oxidation and has a higher rate of respiration than *P. aphthosa*, the species from the more shaded forest habitat. This may indicate that *P. rufescens* thalli experience a higher oxidative stress under normal field conditions. Exposure to UV-B of *P. rufescens* thalli increased SOD activity, while in *P. aphthosa*, UV radiation did not affect SOD activity but increased lipid peroxidation. Thus, the tolerance to oxidative stress of the two species is different, probably because of the dissimilarities in the temperature and light conditions in typical habitats of these species. However, in both species, particularly in *P. aphthosa*, UV-B increased the activity and contribution of energy dissipative alternative respiratory pathway. Such changes in respiration are likely a universal response of lichens to the oxidative stress.

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Antioxidant Activity and Chemical Composition of Extracts from Fruiting Bodies of Xylotrophic Fungi Growing on Birch

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Abstract. The search for new natural sources of biologically active substances is a major issue in pharmaceutical industry. Xylotrophic basidiomycetes are common in forests worldwide, but as a prospective raw source of biologically active compounds they have not been studied as extensively as plants and other groups of fungi. The study is aimed to determine the chemical composition and antioxidant activity of extracts from 10 species of tinder fungi growing on birch and common in the forests in Russia. The chaga muchroom (*Inonotus obliquus*), traditionally used in medicine, was chosen as a standard species. Extracts from fruiting bodies were obtained with water or 95 % ethanol. They contained 4 to 8 types of free amino acids including 2 to 6 essential ones. Perennial basidiocarps were shown to be richer in phenolic compounds and poorer in amino acids than annual ones. Alkaloids and saponins were found in perennial basidiocarps of two species, saponins were also found in annual basidiocarps of one species. Water and alcohol extracts differed in composition and concentration of extractives, and showed different antioxidant (inhibition of lipid peroxidation) and antiradical (ABTS-test, inhibition of NO production) activity. This way it was shown that the nature of the solvent extraction agent is important for the manifestation of biological activity. In most tests, water extracts from chaga showed better antioxidant properties; extracts from *Piptoporus betulinus*

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and *Fomitopsis pinicola* were also effective as antioxidants, which may be promising avenues for future research.

Keywords: xylotrophic fungi, chaga, phenols, antioxidant activity, amino acids, qualitative composition.

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Антиоксидантная активность и химический состав экстрактов ксилотрофных грибов Среднего Урала, произрастающих на березе

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Аннотация. Поиск новых природных источников биологически активных веществ остается актуальной проблемой. Ксилотрофные базидиомицеты широко распространены в лесах, но как сырье для получения биологически активных соединений они менее изучены, чем растения и другие группы грибов. Цель исследования – изучение химического состава и антиоксидантной активности экстрактов 10 видов трутовых грибов, произрастающих на березе и широко распространенных в лесах России. В качестве вида сравнения выбрана чага *Inonotus obliquus*, традиционно используемая в медицине. Экстракцию веществ из плодовых тел проводили водой или 95%-ным этанолом. В экстрактах обнаружено от 4 до 8 типов свободных аминокислот, в том числе от 2 до 6 незаменимых. Показано, что многолетние базидиокарпы богаче фенольными соединениями и беднее аминокислотами, чем однолетние. В многолетних базидиокарпах двух видов были обнаружены алкалоиды и сапонины; также сапонины обнаружены у одного вида с однолетними базидиокарпами. Водные и спиртовые экстракты различались по составу и концентрации экстрактивных веществ и проявляли разную антиоксидантную (ингибирование

перекисного окисления липидов) и антирадикальную (ABTS-тест, ингибирование продукции NO) активность. Таким образом, природа экстрагента имеет значение для проявления биологической активности. В большинстве тестов водные экстракты чаги показали наилучшие антиоксидантные свойства, однако с ними могут быть сопоставимы экстракты *Piptoporus betulinus* и *Fomitopsis pinicola*, что определяет перспективы их дальнейшего изучения.

Ключевые слова: ксилотрофные грибы, чага, фенолы, антиоксидантная активность, аминокислоты, качественный состав.

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Introduction

Industrial development and urbanization affect negatively the biota. Pollution causes oxidative stress in living organisms, which leads to metabolic disorders and pathological conditions in them. This explains high demand in biologically active substances (BAS) with antioxidant and antiradical activity. Artificial synthesis of such compounds in pharmaceutical industry and biotechnology poses a significant challenge, therefore, active search for natural sources of BAS is of great interest.

Higher plants with rich secondary metabolism are major sources of natural BAS. Higher fungi also have a wide range of secondary metabolic pathways, but they have been studied as a potential source of BAS to a much lesser extent than plants. Fungi are known to have been applied in Oriental medicine (Japan, Korea, Vietnam and especially China) and traditional East-European medicine quite a lot, although not as extensively as plants (Lindeqist et al., 2005; Blagodatski et al., 2018). In Russia, the chaga muchroom (*Inonotus obliquus* (Ach. ex Pers.) Pil.) has been long used to treat tumors and inflammations. It has been proven that chaga preparations have a pronounced anticancer and immunomodulatory effect. For this reason, they are used as a prophylactic agent, as well as a component of complementary therapy (Tsai et al., 2017; Gery et al., 2018). In China and Japan, the reishi (also known as lingzhi) mushroom (*Ganoderma lucidum* (Curtis) P. Karst) is widely used for medical treatment. It has been shown that fruiting bodies of ganoderma contain high concentrations of steroids and triterpenes (triterpenic acids and alcohols), which have a pronounced anticancer effect (Zhao et al., 2019). In Japan, extracts of glycoprotein from biotechnologically cultivated *Trametes versicolor* (L.) Lloyd are used as anticancer drugs (Ho et al., 2005). Other types of wood-destroying (tinder) fungi are used in both traditional and modern medicine, but are less studied (Gruendemann et al., 2020; Payamnoor et al., 2020).

Xylotrophic basidiomycetes found in Russian forests are promising for study with respect to content of BAS and their antioxidant and antiradical activity. Considering the fact

that a significant part of Russia is covered with forests, there is a huge potential for raw materials from them. The paper aims to determine the antioxidant and antiradical properties of extracts obtained from fruiting bodies of the most common xylotrophic basidiomycetes and to study their chemical composition qualitatively and quantitatively.

Materials and methods

Biological material

Fruiting bodies of basidiomycetes growing on birch (*Betula pendula* Roth.) were used as material for extraction. They were collected in mixed forest with dominant pine (*Pinus sylvestris* L.) in the vicinity of the Ural Federal University biological station (Russia, Sverdlovsk Oblast, the village of Klyuchi, near the city of Dvurechensk – 56°37'44" N, 61°03'53" E). Fruiting bodies were collected from tree trunks at the height of up to 2 m. Depending on the species abundance, samples were collected from 10–30 trees, 20–50 fruiting bodies each. The material was fixed by heating at 50 °C for 1 h, air-dried to a constant mass, ground and randomized.

Ten species of fungi were studied: *I. obliquus* (the chaga mushroom), *Fomes fomentarius* (L.) Fr., *Fomitopsis pinicola* (Sw.) P. Karst, *Phellinus cinereus* (Niemelä) Parmasto, *Piptoporus betulinus* (Bull.) P. Karst., *Trametes versicolor*, *T. pubescens* (Schumach.) Pilát, *T. gibbosa* (Pers.) Fr., *Trichaptum pergamenum* (Fr.) G. Cunn., *Stereum subtomentosum* Pouzar. The first four species have perennial basidiocarps, the others are annuals. The chaga has been commonly used in traditional and modern medicine and is well studied; for this reason, we used it as a standard species.

Species were identified using identification keys and compared with the species description (Storozhenko et al., 2014; Kotkova et al., 2015). Samples were validated by mycologist, Professor V. A. Mukhin (Ural Federal University, Ekaterinburg).

Extraction

A solvent extraction agent – distilled water or 95 % ethanol (3 mL) – was added to dry biomass of fruiting bodies (150 mg). The mixture was treated with ultrasound for 15 min (570 W) and kept for 25 min in a water bath at 50 °C, occasionally stirred. Then the mixture was centrifuged. Extraction was repeated 3 more times, supernatants were pooled, and volume was adjusted to 15 mL. One milliliter of the obtained extract corresponded to 10 mg of dry biomass (DW) of the fungus.

Antioxidant activity

Antiradical activity was determined by the ability to inhibit the formation of the ABTS-radical – 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) – and was expressed as a percentage; complete inhibition of radical formation was taken as 100 % (Re et al., 1999).

The ability to suppress the formation of nitric oxide was determined in the model with sodium nitroprusside. Complete suppression was taken as 100 %. Negative values indicate the stimulation of nitric oxide production (Umamaheswari, Chatterjee, 2008).

Membrane lipid peroxidation (LPO) was modeled in the reaction of free tween-80 oxidation with atmospheric oxygen in the presence of iron ions, followed by determination of formed malondialdehyde in the reaction with thiobarbituric acid. Complete suppression of tween-80 oxidation was taken as 100 %. Negative values correspond to prooxidant activity of the extract and stimulation of LPO processes (Umamaheswari, Chatterjee, 2008).

The total reduction potential was determined by the formation of molybdenum

blue. The absence of reducing power was taken as 0 conventional units. Positive values indicated the reduction potential of the examined extracts (Umamaheswari, Chatterjee, 2008).

Qualitative analysis of the chemical composition

To detect alkaloids, precipitation reactions were carried out with iodide complexes of mercury, bismuth, cadmium, phosphotungstic, phosphoromolybdic salts and picric acids, tannin, and iodine (Sorescu et al., 2018). The reactions were carried out by the micro method using an immunological plate. The presence of alkaloids was justified if the analytical effect was observed for 4 or more reactions out of 8 carried out.

The presence of phenols was determined by the reaction with iron (III) chloride; flavonoids were identified by the Synod-test (reduction with molecular hydrogen to red flavones in an acidic medium), by increased yellow coloring with lead acetate in an alkaline medium (Shaikh, Patil, 2020), and by aluminum chloride test (Sheel et al., 2014).

Saponins were determined by the formation of foam upon shaking with water (Sorescu et al., 2018) and formation of a precipitate with lead acetate (Pavlovskaya et al., 2012).

Paper chromatography

For paper chromatography, an aliquot of the extract equivalent to 4 mg DW of fungi was applied onto chromatographic paper. Separation was carried out by descending chromatography in 95 % ethanol for 15 h. Chromatograms were dried and sprayed with 0.2 % ninhydrin in acetone. Amino acids were identified by comparing the Rf values of chromatographically separated substances and standards.

Phenolic compounds were determined after the exposure to UV irradiation before and after treatment with ammonia vapor. Phenols were identified based on the color of the spot, its fluorescence and a change in color intensity. Phenolic acids and alcohols fluoresced in blue light. Polyphenols were detected as gray and brown spots (Sokolova et al., 2018).

Quantitative analysis of the composition of extracts

The content of phenolic compounds was determined spectrophotometrically with the Folin-Chocalteu reagent, with gallic acid as the standard (Larayetan et al., 2019).

Flavonoids were determined spectrophotometrically as complexes with aluminum chloride, with rutin as the standard (Larayetan, 2019).

Free amino acids were determined spectrophotometrically with the ninhydrin reagent, with glycine as the standard (Kotova et al., 2020).

All quantitative measurements were performed using an Infinite Tecan M 200 Pro microplate spectrophotometer (Tecan, Austria).

Statistical data processing

Quantitative analyzes were done in 4–5 (identification of phenols, flavonoids, amino acids) or 6–8 (antioxidant reactions) analytical replicates. Statistical significance of the differences was determined by the nonparametric Mann-Whitney *U*-test using the Statistica 8.0 and MS Excel 2013 software packages. The values are presented as the arithmetic mean and its standard error (SE).

Results and discussion

Antioxidant activity of extracts

An assay of antioxidant activity was carried out in water and ethanol extracts from 10 xylotrophic fungi species growing on birch in the forests of the Middle Urals (Table 1). The reducing power of the extracts varied depending both on the species and type of the solvent extraction agent.

Table 1. Antioxidant activity of water and ethanol extracts from fruiting bodies of tinder fungi

Species	Reduction potential, c. u.		Inhibition of LPO, %	
	Ethanol	Water	Ethanol	Water
I. obliquus	14.0±2.8	118.4±13.3^{x}	9.4±2.8	43.4±3.9^{x}
F. fomentarius	123.3±7.9*	48.6±6.9*^{x}	16.1±11.0	3.9±2.0*^{x}
F. pinicola	52.4±3.4*	23.8±1.1*^{x}	21.2 ±7.0*	3.3±2.0*^{x}
P. betulinus	129.0±5.0*	115.2±8.1	-0.5±1.0*	-8.0±2.4*^{x}
P. cinereus	11.9±6.4*	32.0±2.6*^{x}	13.1±2.3	1.3±1.0*^{x}
S. subtomentosum	47.4±2.7*	73.9±2.9*^{x}	-5.1±2.5*	1.3±1.0*
T. gibbosa	26.2±5.6*	28.8±12.0*	-4.1±2.5*	-4.1±3.4*
T. pergamenum	40.8±8.3*	59.7±8.3*	-1.2±2.0*	-4.6±2.0*
T. pubescens	31.3±4.8*	63.6±20.0	-10.4±0.2*	9.5±0.6*^{x}
T. versicolor	18.8±3.2*	68.0±6.1*^{x}	-1.9±1.5*	-10.0±5.0*

Note. Results are expressed as mean ± SE. Values marked by special symbols are significantly different ($p < 0.05$) from other related values: *from chaga (*I. obliquus*), ^{x}from ethanol.

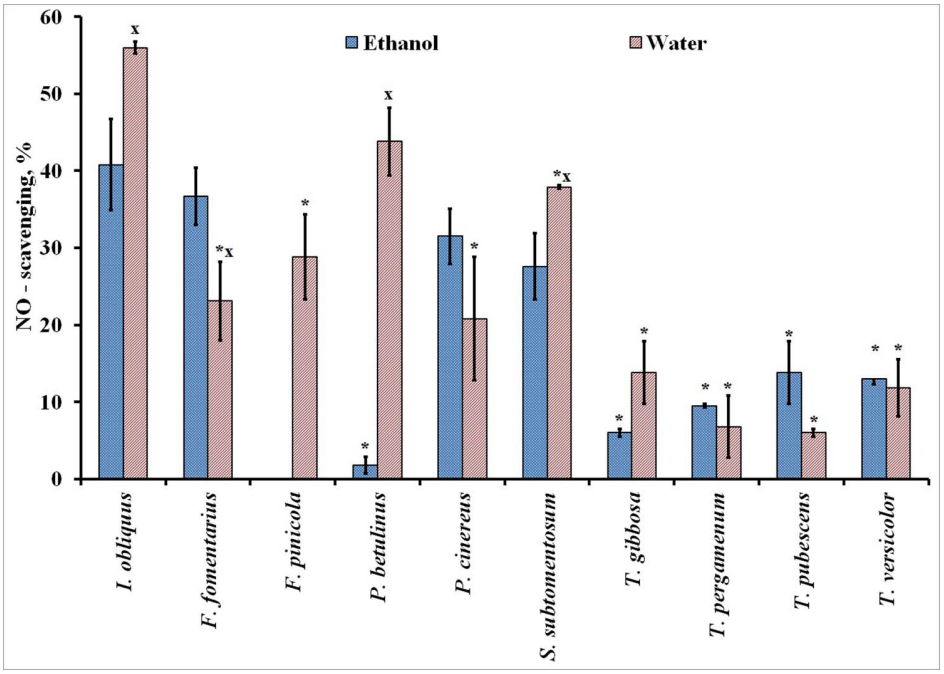
Water extracts from the chaga, *T. versicolor* and *P. cinereus* had greater reducing power than ethanol ones (8.4 times, 3.6 times and 2.7 times greater, respectively), while water extracts from *F. fomentarius* and *F. pinicola* were 2.5 times less active than ethanol ones. For the other species, reducing power of aqueous and alcoholic extracts differed less markedly. A comparison of water extracts from different species showed that the chaga and *P. betulinus* had the highest reducing power. Ethanol extracts from *T. versicolor* and *P. cinereus* practically did not differ from ethanol chaga extracts in terms of their reduction potential. Ethanol extracts from other species showed a significantly greater reducing power than those from the chaga. *P. betulinus* ethanol extract showed the highest activity. It was 9 times more active than chaga extract. Thus, the highest reducing power was found in water chaga extract, and water and ethanol extracts from *P. betulinus* (Table 1).

Membrane lipid peroxidation (LPO) is one of the nonspecific responses in cells under oxidative stress. Water and ethanol extracts from the investigated fungi were tested as antioxidants in the LPO model. Among water extracts, the best result was shown for chaga – the amount of malondialdehyde (MDA) formed in the LPO reaction was decreased by 43 % in the presence of chaga extract. Water extract from *T. pubescens* inhibited lipid peroxidation by 9.5 %, while water extracts from the other species either did not suppress the formation of MDA, or even stimulated it, i. e., they were prooxidants (Table 1).

Ethanol chaga extract inhibited the formation of MDA to a lesser extent (9.5 %) than its aqueous extract. Extracts from *F. fomentarius* and *P. cinereus* showed approximately the same efficiency (16 and 13 %). Ethanol extract from *F. pinicola* was more active than the water one (21 % versus 3 %). The rest of the samples revealed a slight prooxidant effect and stimulated formation of MDA by 1–10 %.

Thus, not all samples with high reduction potential inhibited the formation of MDA. Some of them had a prooxidant effect. In some cases, fungal extracts could both stimulate and inhibit the formation of MDA, depending on the solvent used (Table 1).

In addition to reactive oxygen species, oxidative stress can be caused by reactive



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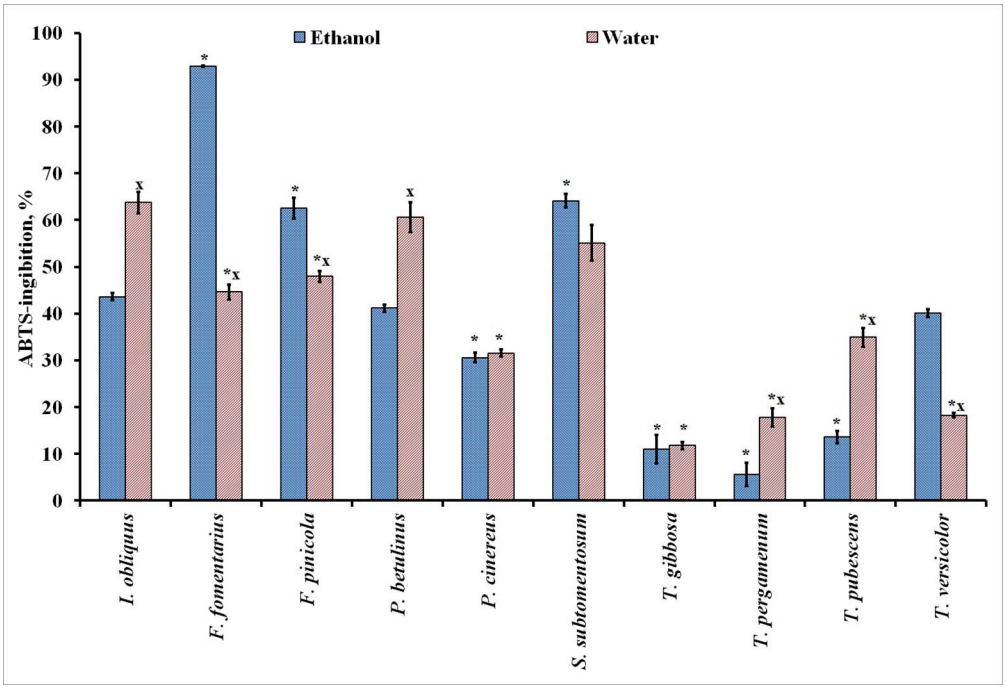

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Fig. 1. Antiradical activity of extracts from fruiting bodies of tinder fungi: inhibition of NO (a) and ABTS-radicals production (b). Results are expressed as mean ± SE. Values marked by special symbols are significantly different ($p < 0.05$) from other related values: *from chaga (*I. obliquus*), ^{x}from ethanol

nitrogen species (Brieger et al., 2012). An analysis of the extracts' ability to inhibit nitrogen oxide production *in vitro* showed that the aqueous chaga extract had the highest effect – NO production was decreased by 56 % (Fig. 1a). The extracts from *P. betulinus* and *S. subtomentosum* showed a slightly lower activity (43.8 % and 37.9 % decrease in NO production, respectively). Water extracts from other fungi also inhibited NO production, but they were at least 2 times less effective than the chaga extract.

Ethanol extracts inhibited the formation of nitric oxide to a lesser extent than water ones. The highest inhibitory activity was shown by ethanol extracts from *I. obliquus* (40.8 %), *F. fomentarius* (36.7 %) and *P. cinereus* (31.5 %) (Fig. 1a).

In the test for suppression of the ABTS radical production, the water chaga extract was 1.5 times more active than its ethanol extract, in contrast to *F. fomentarius*; its aqueous extract was 2 times less active than the ethanol one (Fig. 1b). This corresponds to the reducing power of extracts (Table 1). The activity of *F. fomentarius* ethanol extract was maximal in comparison with other species (about 90 %) and exceeded the activity of the water chaga extract by 1.5 times. *F. pinicola*, *P. betulinus*, *S. subtomentosum* showed antiradical activity to be comparable to that of chaga. Extracts from the other species were significantly inferior to chaga in terms of this effect (Fig. 1b).

Chemical composition of extracts from fruiting bodies of xylotrophic basidiomycetes

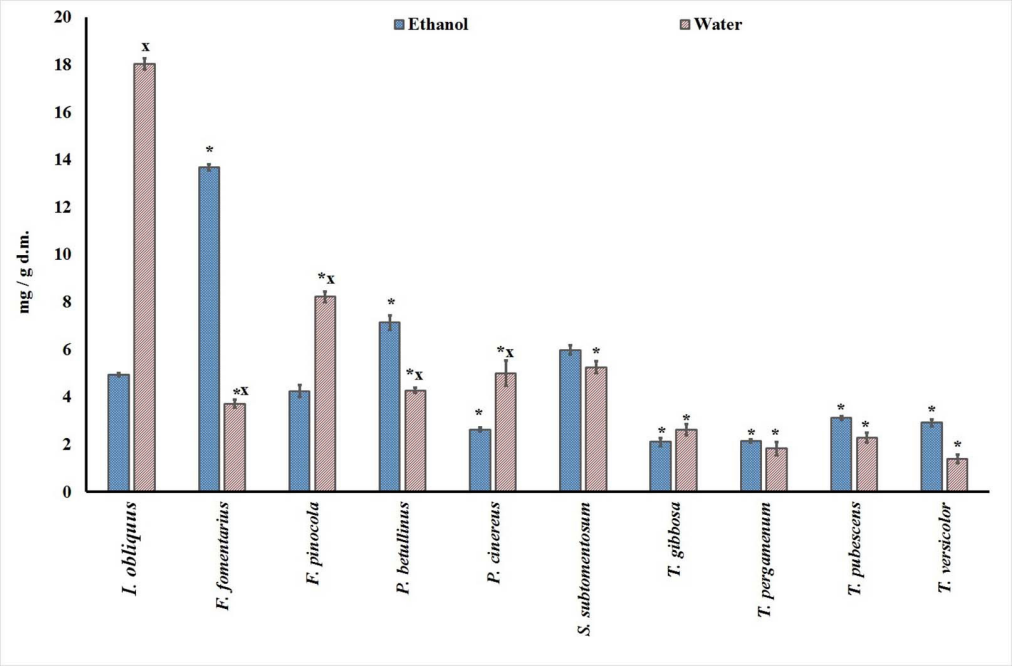
Antioxidant activity in different fungi may be associated with production of different compounds. Previously, using gas chromatography combined with mass spectrometry we showed that methanol extracts from xylotrophic fungi contain more than 100 minor and basic compounds, including free carbohydrates arabinitol and sorbitol, hexa- and octodecanoid acids and their derivatives, ergosterol and its derivatives, lupeol and other secondary compounds (Kurchenko et al., 2020). The results of quality tests for biologically active substances in the studied extracts are presented in Table 2. Metabolite composition of aqueous and ethanol extracts from the same species was similar.

Alkaloids were found only in two species, *F. fomentarius* and *F. pinicola*. *F. fomentarius*, *P. betulinus* and *F. pinicola* showed the presence

Table 2. The qualitative composition in extracts from fruiting bodies of tinder fungi (similar in water and ethanol extracts)

Group of compounds	Fungi species									
	I. obliquus	*F. fomentarius*	*F. pinicola*	*P. betulinus*	*P. cinereus*	*S. subtomentosum*	*T. gibbosa*	*T. pubescens*	*T. versicolor*	*T. pergamenum*
Alkaloids	-	+	+	-	-	-	-	-	-	-
Flavonoids	-	+	-	-	-	-	-	-	-	-
Saponins	-	+	+	+	-	-	-	-	-	-
Simple phenols	+	+	+	+	+	-	-	-	-	-

Note. (+) compound is determined; (–) compound is not determined



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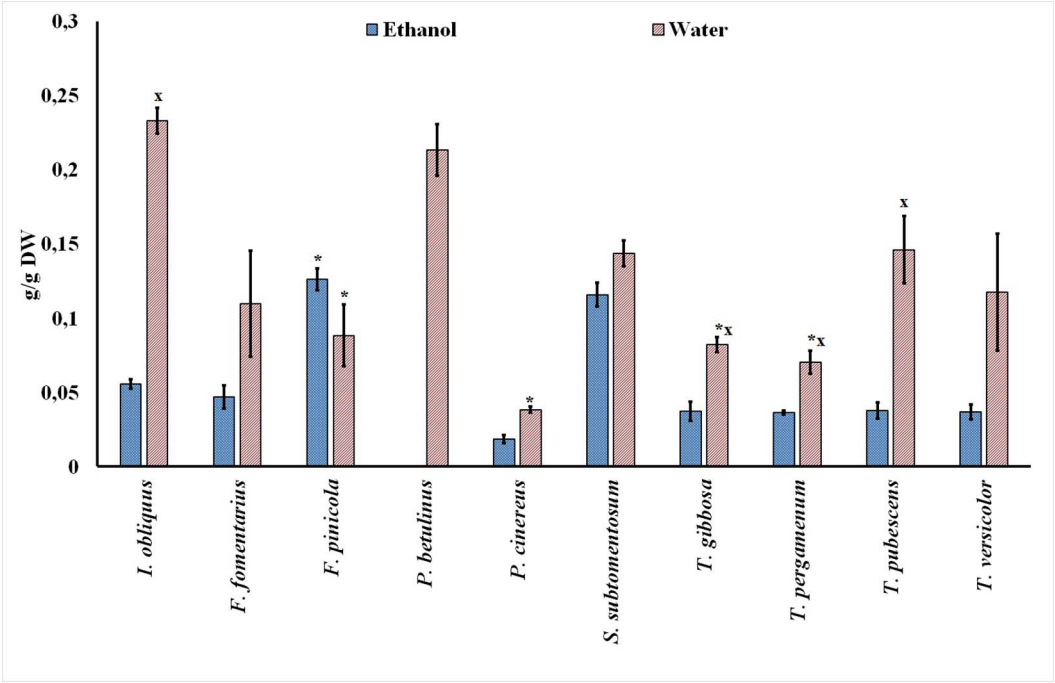

b

Fig. 2. Phenols (a) and extractives (b) in water and ethanol extracts from fruiting bodies of tinder fungi. Results are expressed as mean ± SE. Values marked by special symbols are significantly different ($p < 0.05$) from other related values: *from chaga (*I. obliquus*), [x]from ethanol

of saponins. Alkaloids and saponins usually have a pronounced biological activity. It makes the species rich in these compounds promising for further study, in spite of the fact that their content is not directly related to the antioxidant ability of drugs. Antiradical and antioxidant activity of extracts is manifested to a greater extent due to phenolic compounds, including flavonoids. Qualitative reactions showed the presence of flavonoids in *F. fomentarius*. In other species, they were not detected. However, we cannot exclude that they might be present in trace amounts, out of the detection limits of the methods used. Phenols were found only in the extracts from perennial basidiocarps and *P. betulinus*, while in annual basidiocarps they were not discovered, or their concentration might be lower than the detection limits of the tests (Table 2). This difference might be related to the life forms of the fungi. Species with perennial basidiocarps have to withstand many unfavorable environmental factors, therefore, they accumulate phenols that perform an antioxidant function and protect against animals and parasites. Antioxidant ability of these species is also justified by the presence of alkaloids.

Quantitative analysis showed that the total content of phenols differed in water and ethanol extracts (Fig. 2a). In chaga water extract, phenols concentration was almost 4 times higher than in the ethanol one. It is explained by the fact that chaga is rich in melanins, which are phenolic compounds soluble in water, but not in ethanol (Sushinskaya, Kurchenko, 2006). In *F. pinicola*, water extract also contained more phenols than alcoholic extract – 0.8 %. Water chaga extract contained more phenols than any other samples – 2 % of dry mass. Alcoholic extracts from *F. fomentarius* and *P. betulinus* contained 1.3 % and 0.8 % of phenols, respectively. In alcoholic extracts from the other species, there were no more than 0.5 % of phenols. Thus, water can be recommended for extraction of phenolic compounds from *I. obliquus*, *F. pinicola* and *P. cinereus*, and in the case of *F. fomenterius*, *P. betulinus*, and *T. versicolor*, phenols are extracted more efficiently with ethanol.

The qualitative composition of phenols was compared based on chromatography data (Table 3). Chaga, *F. pinicola*, and *P. betulinus* had the greatest variety of phenols: they contained at

Table 3. Chromatography of phenols in extracts from fruiting bodies of tinder fungi (similar in water and ethanol extracts)

Species	Number of compounds	Group of compounds		
		Phenolic alcohols and acids	Polyphenols	Aurons
I. obliquus	8	5	2	1
F. fomentarius	5	3	2	0
F. pinicola	8	4	4	0
P. betulinus	7	2	5	0
P. cinereus	4	4	0	0
S. subtomentosum	4	4	0	0
T. gibbosa	4	3	1	0
T. pubescens	4	4	0	0
T. versicolor	1	0	1	0
T. pergamenum	2	1	1	0

least 7–8 individual compounds. The rest of the species contained 4–5 compounds; *T. versicolor* and *T. pergamenum* were poorer in phenol diversity – 1 and 2 spots, respectively. Species with a high total amount of phenols had a greater variety of compounds. Solvents used for chromatography of chaga extracts did not allow to separate melanins and they remained on the starting line. According to our data, phenolic acids and alcohols in chaga prevailed over polyphenols, excluding melanins. One orange fluorescent spot was identified as auron. It was not found in other species. In *F. pinicola*, phenolic alcohols and acids accounted for half of identified substances, and polyphenols accounted for the other half. In *P. betulinus*, on the contrary, polyphenols prevailed over phenolic acids and alcohols.

Flavonoids are a special group of polyphenols. The most common source of them are plants, but there is evidence of their presence in fruiting bodies of fungi (Kim et al., 2008; Payamnoor et al., 2020). Quantification (Table 4) revealed flavonoids in *F. fomentarius* – 0.5 mg/g DW in ethanol extract, and in water extract from chaga its concentration was about 0.2 mg/g DW. In the other species, the concentration of flavonoids did not exceed 0.05 mg/g DW.

While antioxidant activity of phenolic compounds is widely discussed in literature, amino acids are less often considered as antioxidants, although some of them contain aromatic and sulfhydryl groups potentially providing antioxidant activity. The concentration of amino acids in the extracts varied from 1.6 to 95.9 mg/g DW, depending on the species and solvent extraction agent (Table 4). The highest amount of free amino acids was found in *P. betulinus*: 3 % in water and 9.5 % in ethanol extracts. Among the studied species, only this one is considered conditionally edible in several East-European countries (Pleszczyńska et al., 2017). Unlike other species, it has soft, yet rather fleshy annual basidiocarps, with a high content of free amino acids. This species also showed high antioxidant activity and reducing power (Table 1, Fig. 1). In general, the content of free amino acids in the extracts was higher than that of phenolic compounds as amino acids are primary metabolites and precursors for the biosynthesis of secondary compounds.

Table 4. Concentration of amino acids and flavonoids in extracts from fruiting bodies of tinder fungi

Species	Amino acids, mg/g DW		Flavonoids, μg/g DW	
	Ethanol	Water	Ethanol	Water
I. obliquus	1.6±0.5	13.9±0.6^{x}	31.8±3.0	165.9±8.3^{x}
F. fomentarius	7.8±0.4*	7.6±5.3	453.8±22.5*	15.7±2.0*^{x}
F. pinicola	8.6±0.9*	9.6±2.2	45.8±4.1	N.d.*
P. betulinus	95.9±5.1*	31.8±3.9*^{x}	49.7±4.5	45.0±4.0*
P. cinereus	2.3±0.3	7.0±1.5^{x}	37.3±3.1	25.2±2.0*
S. subtomentosum	20.7±0.9*	21.4±0.2*	58.2±5.4*	N.d.*^{x}
T. gibbosa	14.2±0.9*	7.4±5.9	10.9±1.0*	N.d.*^{x}
T. pergamenum	12.1±1.7*	12.2±2.7	N.d.*	N.d.*
T. pubescens	31.1±4.2*	21.4±3.4*	9.3±1.0*	14.6±1.5*
T. versicolor	6.8±1.7*	14.3±3.7	7.3±0.7*	5.9±1.0*

Note. Results are expressed as mean ± SE. N.d. – the compound was not detected. Values marked by special symbols are significantly different ($p < 0.05$) from other related values: *from chaga (*I. obliquus*), ^{x}from ethanol.

The comparison of antioxidant activity of the extracts (Table 1, Fig. 1) and the content of metabolites (Table 4, Fig. 2a) did not reveal a direct relationship between these characteristics. For example, a decrease in the content of phenols in aqueous extract from *F. fomentarius* led to a decrease of NO-scavenging and ABTS-inhibition, while for *P. betulinis* it led to an increase in these values. For *P. betulinus*, antioxidant activity, probably, was associated not with phenolic compounds, but with some other group of substances, perhaps amino acids that this species was rich in.

The nutritional value of different amino acids varies, so it is important to qualify them. Table 5 shows the results of paper chromatography of free amino acids. No difference was found in the composition of water and ethanol extracts, so the table includes unified data. The fruiting bodies of fungi contained from 4 to 8 individual amino acids, including essential ones. All investigated species contained non-proteinogenic amino acid ornithine due to the ornithine cycle as the way of urea synthesis in fungi. Among the essential amino acids, lysine was found in all species and cysteine was found in all except chaga. Methionine, leucine, phenylalanine, and tryptophan were rarely found. Histidine was not detected at all. Among the nonessential amino acids, alanine and arginine were detected. A sulfur-containing amino acid, cysteine was found in all fungi, excluding chaga; tyrosine, containing a phenyl radical was found in 8 species. These amino acids can act as antioxidants, as well as chelating agents for heavy metals. This confirms the assumption that antioxidant and antiradical activity of the extracts may be associated not only with phenolic compounds, but also with free amino acids. *P. betulinus*, which had the highest content of free amino acids, turned out to be one of the poorest in terms of the variety of essential amino acids – only 4 of them were identified. However, three of them possess antioxidant activity.

Table 5. The qualitative composition of amino acids in extracts from fruiting bodies of tinder fungi (similar in water and ethanol extracts)

Species	Essential									Nonessential			Total
	Leucine	Lysine	Methionine**	Cysteine **	Phenylalanine*	Tyrosine*	Tryptophan*	Valine	Total number	Alanine	Arginine	Ornithine	
I. obliquus		+				+			2	+		+	4
F. fomentarius		+		+		+	+	+	5	+	+	+	8
F. pinicola		+		+	+		+		4	+	+	+	7
P. betulinus		+	+	+		+			4	+	+	+	7
P. cinereus		+	+	+		+		+	5		+	+	7
S. subtomentosum		+		+		+		+	4	+	+	+	7
T. gibbosa	+	+		+		+		+	5	+	+	+	8
T. pubescens	+	+		+	+	+		+	6		+	+	8
T. versicolor	+	+	+	+					4	+	+	+	7
T. pergamenum	+	+		+		+			4	+	+	+	7

Note. * – aromatic amino acids; ** – sulfur-containing amino acids

The amount of extractives is an integral indicator that is often used to characterize biological raw materials when the chemical composition of the object has not been sufficiently investigated. This fully applies to fungi. For chaga, for example, it is known that the biological activity of extracts is higher, the higher the concentration of extractives in them is (Sushinskaya, Kurchenko, 2006). The data on the amount of extractable substances are presented in Fig. 2b. More extractives were found in water extracts compared to ethanol ones. Among the water extracts, those with a higher content of extractable substances had a higher antioxidant activity. When comparing annual and perennial basidiocarps, it was noted that the variations in total extractives were less pronounced than in the concentration of phenols, amino acids and antioxidant activity. Extracts from annual basidiocarps contained more amino acids and less phenols and flavonoids than perennial ones that led to lower antiradical activity in the ABTS test, lower ability to suppress NO production and to inhibit lipid peroxidation in comparison with extracts from perennial fruiting bodies.

Conclusion

The study of the extracts from fruiting bodies of xylotrophic fungi showed that they are a promising raw material for obtaining substances with high antioxidant activity. Species with perennial basidiocarps were characterized by a greater diversity and a higher content of phenolic compounds and had a higher antioxidant activity than species with annual fruiting bodies.

Among species, the chaga mushroom, which is traditionally used in medicine, revealed the best antioxidant capacity and the highest concentration of phenols in water extracts. The highest level of phenols in ethanol extracts was found in *F. fomentarius*. Other investigated species were also shown to vary in chemical composition and antioxidant activity of water and ethanol extracts. Extracts from *P. betulinus* fruiting bodies were rich in free amino acids, which makes this species a promising raw material for food additives. Extracts from this fungus also showed high antiradical and antioxidant activity. *F. fomentarius* and *F. pinicola*, which contained alkaloids and saponins, also need further study for practical use. All examined species, except for *F. fomentarius*, did not contain flavonoids, or their content was below the detection limit.

Based on the above results it can be concluded that some of the xylotrophic fungi species growing on birch may be of interest as a potential natural source of antioxidants and free amino acids.

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High Temperatures Induce ROS Generation and Damage to Respiratory Activity in *Saccharum officinarum* Suspension Cells

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Abstract. High temperatures are important abiotic stressors affecting plant growth, development and productivity. One of the consequences of unfavourable temperature effects on plants is an increase in reactive oxygen species (ROS) generation. However, what role ROS will play in the further fate of the cell under temperature stress depends on many external and internal factors. Therefore, the aim of this study was to identify the relationship between ROS content and mitochondrial function in the cells of a *Saccharum officinarum* suspension culture under high temperatures. The work was carried out using fluorescence microscopy and the polarographic analysis method. We found the most significant increase in ROS content in *S. officinarum* cells during temperature treatments (that did not cause immediate cell death in culture) was at 45 and 50 °C. The ROS content was largely determined by mitochondrial activity, as evidenced by a decrease in the electrochemical potential on the inner mitochondrial membrane ($\Delta\Psi_m$), and a simultaneous decrease of ROS levels in cells under the carbonyl cyanide m-chlorophenyl hydrazine (CCCP) treatment. The decrease in the respiratory activity of cells under high temperatures was determined by the decrease of the cytochrome pathway (CP) contribution. It should be noted that the reduction in respiration rate at a temperature of 50 °C preceded the death of cells in the culture, and was not a consequence of it.

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Keywords: *Saccharum officinarum*, high temperature stress, reactive oxygen species, electrochemical potential on the inner mitochondrial membrane, carbonyl cyanide m-chlorophenyl hydrazine, respiration.

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Повышенные температуры вызывают образование АФК и нарушения дыхания в клетках суспензионной культуры *Saccharum officinarum*

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Аннотация. Высокие температуры являются важными абиотическими стрессорами, влияющими на рост, развитие и продуктивность растений. Увеличение образования активных форм кислорода (АФК) – одно из последствий их негативного влияния. Однако то, какую роль сыграют АФК в дальнейшей судьбе клетки в условиях температурного стресса, зависит от множества внутренних и внешних факторов. Таким образом, целью данной работы стало выявление взаимосвязи между содержанием АФК и функционированием митохондрий в клетках суспензионной культуры *Saccharum officinarum* при действии повышенных температур. Данное исследование проводилось с использованием флуоресцентной микроскопии и полярографического анализа. Было обнаружено, что температуры 45 и 50 °С вызывают значительное увеличение содержания АФК в клетках *S. officinarum*, что, тем не менее, не приводит к немедленной гибели клеток в культуре. Содержание АФК во многом определялось активностью митохондрий, о чем свидетельствует снижение электрохимического потенциала на внутренней митохондриальной мембране ($\Delta\Psi_m$) и одновременное снижение уровня АФК в клетках при обработке карбонилцианид-м-фенилгидразоном (СССР). Уменьшение дыхательной активности в клетках при высокотемпературном воздействии было обусловлено снижением вклада цитохромного пути (ЦП). Следует отметить, что снижение скорости дыхания при температуре 50 °С предшествовало гибели клеток в культуре, а не было ее следствием.

Ключевые слова: *Saccharum officinarum*, высокотемпературный стресс, активные формы кислорода, электрохимический потенциал на внутренней митохондриальной мембране, карбонилцианид-м-фенилгидразон, дыхание.

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Introduction

Temperature is one of the most important environmental factor affecting the growth, development and productivity of plants. Since plants are sessile and cannot avoid stressful effects, they have formed various evolutionary physiological and biochemical adaptation mechanisms that ensure their survival under adverse conditions. High temperatures cause changes to cell membrane lipid contents, resulting in changes in physical and chemical characteristics (Djanaguiraman et al., 2018). They induce synthesis of specific proteins (heat stress proteins and dehydrins), secondary metabolites (phenolic and terpenoid compounds) and compatible osmolytes (water soluble sugars, polyoles, proline and glycine betaines) (Vierling, 1991; Graether, Boddington, 2014; Nievola et al., 2017). All these processes require significant energy costs, so the need for ATP increases under stress conditions.

Mitochondria are energy supply organelles in both heterotrophic and photosynthetic plant cells. The mitochondrial electron-transport chain (ETC) creates electrochemical potential on the inner membrane ($\Delta\Psi_m$) for the synthesis of ATP by ATP-synthase. The cytochrome pathway (CP) of respiration in plants (as in animals) contains four main complexes: NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV). Electron transport in complexes I and III is accompanied by ROS generation (Møller, 2001). There is a superoxide anion ($O_2^{\bullet-}$), which can be converted to H_2O_2 by a matrix-localised manganese superoxide dismutase (Morgan et al., 2008).

Under normal conditions, ROS are important secondary messengers and participants of plant cell signalling pathways, playing an important role in the integration and regulation of cellular metabolism, as well as in the formation of plant responses to environmental factors (Kohli et al., 2019). However, ROS are not only signal transduction molecules, but also toxic, highly active compounds. An increase in their content in cells above a critical level can cause the development of intracellular oxidative stress, accompanied by damage to lipids, proteins and nucleic acids (Kolupaev, Karpets, 2009; Dmitrieva et al., 2018; Tripathi et al., 2020).

Under adverse conditions, the formation of plant acclimatisation mechanisms to stress factors is very often accompanied by fluctuation in the energy status of cells and changes to the respiration process (Baena-González, Sheen, 2008). This can lead to an intensification of ROS generation in mitochondrial ETC. To

protect respiratory metabolism, and to prevent over-reduction of ETC components, plant mitochondria contain an alternative pathway (AP) of respiration as well as the classical CP. This involves rotenone-insensitive II type NAD(P)H dehydrogenases, ubiquinone and alternative cyanide-resistant oxidase (AOX) (Wanniarachchi et al., 2018). Electron flow via the AP is not coupled with proton pumping, and energy obtained during substrate oxidation is dissipated as heat. ROS production decreases as the AP bypasses complexes I and III (the main points of ROS generation) (Noctor et al., 2007; Popov, 2014).

AOX is an additional terminal oxidase in plant mitochondria. It is located on the matrix side of the inner mitochondrial membrane and transfers electrons from ubiquinone to oxygen, bypassing two of the three points of electron and proton transport coupling. It has been shown that AOX is activated by limitation of CP electron transport and an elevated $NADH/NAD^+$ ratio (Vanlerberghe, 2013). It has been stated that the role of AOX in plant respiratory metabolism significantly increases under stress conditions. High and low temperatures and drought can all lead to AOX activation (Bartoli et al., 2005; Grabelnych et al., 2014; Borovik, Grabelnych, 2018). It is possible that one of the functions of AOX is regulation of ROS production (Popov, 2014). This is supported by the fact that AOX is activated by mitochondrial thioredoxins that are involved in protecting these organelles from the toxic effects of ROS (Martí et al., 2009).

Suspension cell cultures of plants are practical, and are therefore frequently used as model objects for investigating plant cell responses to stress (Nguyen et al., 2016) and the processes of cell differentiation and death (Zavala-Ortiz et al., 2020). Despite the fact that the effect of high temperatures on plant metabolism is frequently studied today, including using suspension cell cultures (Rikhvanov et al., 2007), studies dealing with respiratory metabolism in plant suspension cell cultures under these conditions are very few. There are a number of studies regarding respiration in plant suspension cell cultures that are under stress caused by a lack of nutrients (Sieger et al., 2005) or the action of chemical agents, e. g. H_2O_2 (Robson, Vanlerberghe, 2002). However, the relationship between respiratory metabolism and ROS generation in the cells of suspension cultures is not well understood (Fedyaeva et al., 2014). Using an *Arabidopsis thaliana* cell culture, it has been shown that temperatures close to 40 °C, i. e. 37 and 39 °C, acted on cells as a mild heat shock and promoted the formation of induced thermotolerance mechanisms (Rikhvanov et al., 2007). Our purpose was to study the effects of a hard heat shock on various parameters of cellular metabolism. Therefore, a number of high temperatures, from 45 to 60 °C, were investigated. Considering the high thermotolerance of *Saccharum officinarum*, the cell culture of this species is a good model for studying the effect of high temperatures on the energy metabolism of plant cells and the role of cellular respiration under stress.

This study has shown the relationship between respiratory activity, $\Delta\Psi_m$ and ROS levels in *S. officinarum* suspension culture cells for the first time. Differences between the various respiratory pathways functioning under high-temperature treatments that caused cell death in culture and did not have a lethal effect have been revealed. It has been shown that high temperatures caused ROS production to increase. This effect was dependent on the duration of treatment exposure and mitochondrial activity. High temperatures that led to cell death caused significant respiratory depression, mainly due to inhibition of the CP.

Materials and methods

Plant material, growth conditions and high temperature treatments

We used a suspension cell culture of *Saccharum officinarum* (cultivar POJ2878, line resistant to anoxia, obtained at IPP RAS and kindly provided by Shmakov V. N. (PhD, Senior Researcher of the Laboratory of Plant Genetic Engineering, SIPPB SB RAS)). Cell culture was grown using an MS medium, containing sucrose (36 g/L), nicotinic acid (0.6 mg/L), pyridoxine (0.6 mg/L), thiamine (1.2 mg/L), 2.4-D (3 mg/L), inositol (120 mg/L) and potassium dithiocarbamate (6 mg/L). The culture was placed in a fresh medium every 14 days with a 1:7 dilution. The culture was grown in 200-mL Erlenmeyer flasks, in the dark and in a thermostatic chamber at 26 °C with constant stirring on a rotary shaker at 130 rpm. After eight days of cultivation, the culture was subjected to high temperature treatments. The treatments (45, 50, 55 and 60 °C) were carried out using a mini thermal shaker (TS-100, «BioSan», Latvia) and a water thermostatic shaker («Elpan», Poland). After each treatment, the culture was returned to controlled conditions (26 °C) for one, two or four days.

Microscopic analysis

Microscopic analyses of cells were performed using a light microscope (Axiostar plus, Carl Zeiss, Gottingen, Germany) and an inverted fluorescent microscope (AxioObserver Z1, Carl Zeiss) with a digital monochrome camera (AxioCam MRm3). The software package AxioVisionRel4.6 was used to capture and analyse images. We used the following filters: filter set 15 (EX BP 546/12, BS FT 580 and EM LP 590) and filter set 10 (EX BP 450–490, BS FT 510 and EM BP 565).

Determination of cell survival

The proportions of living and dead cells in the cultures were determined by double staining with fluorescent probes: vital fluorescein diacetate (FDA, 50 μM) and lethal propidium iodide (PI, 7 μM) (Lyubushkina et al., 2014b). Briefly, 100 μL of culture was placed in 2 mL microcentrifuge tubes and incubated for 2 min at 26 °C (with the addition of dyes) on a TS-100 mini thermal shaker.

ROS and $\Delta\Psi_m$ detection

To detect ROS levels in the cells of the *S. officinarum* culture, we used a fluorescent sensor (H_2DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate, 1 μM) according to the method described earlier (Lyubushkina et al., 2014a). Briefly, 100 μL of culture was placed in 2 mL microcentrifuge tubes, and incubated for 10 min at 26 °C (control) or at 45, 50, 55 or 60 °C (for heat treatments) with the addition of dye on a TS-100 mini thermal shaker.

For qualitative visualisation of the $\Delta\Psi_m$, we used the potential-dependent fluorescent cationic sensor JC-1 (5.5'.6.6'-tetrachloro-1.1'.2.2'-tetraethylbenzimidazolo-carbocyanine, 20 μM) according to the method described previously (Lyubushkina et al., 2014a). Briefly, 100 μL of the culture was placed in 2 mL microcentrifuge tubes. Then 100 μL of staining buffer (25 mM MES, 2 % glycerol, pH 5.5) was added and the mixture was incubated for 10 min at 26 °C (control) or at 45, 50, 55 or 60 °C (heat treatments) with the addition of dye.

In some experiments studying ROS and $\Delta\Psi_m$ in *S. officinarum* cell culture we used carbonyl cyanide m-chlorophenyl hydrazine (CCCP, 20 μM), which is a protonophore, and is used to detect mitochondrial participation in ROS and $\Delta\Psi_m$ generation processes.

Cell respiration analysis

The rate of oxygen uptake by the *S. officinarum* suspension culture cells was measured at 26 °C using the «Oxytherm»

system (Hansatech Instruments, Great Britain) with a Clark-type oxygen electrode. We used a sequential addition of potassium cyanide (1.2 mM, KCN, an inhibitor of cytochrome *c* oxidase) and benzhydroxamic acid (3 mM, BHAM, an inhibitor of cyanide-resistant alternative oxidase) to measure the CP and AP capacities, respectively. The oxygen uptake recorded after addition of inhibitors was considered nonspecific and was not included in the respiratory activity. The optimal concentrations of inhibitors were determined by titrating the suspension cell culture with increasing concentrations of the inhibitors to saturate the O_2 uptake. The oxygen concentration in air-saturated water at 26 °C was assumed to be 253 μM. The respiration rate of cells was calculated as nmol O_2/(g fresh weight (FW) × min).

Statistical analysis

At least three independent experiments were performed with 2–8 repeats. Data are presented as medians (Q_{50}) and error bars are the Q_{25} and Q_{75} percentiles. To visualise ROS content and $\Delta\Psi_m$ data we used a box-and-whiskers diagram. The line in the «box» is Q_{50}, and the borders of the «box» limit the interquartile range between Q_{25} and Q_{75}. The «whiskers» represent maximum and minimum values. The Shapiro–Wilk test was used as the normality test. Kruskal–Wallis one-way analysis of variance on ranks was used. All pairwise multiple comparison procedures were performed using Dunn's method (n = 3–10). Significant differences ($p < 0.05$) at Q_{50} are marked with different letters next to the corresponding point or the column on the graphs.

Results

High temperatures induce cell death in S. officinarum suspension culture

There were 80–85 % of cells alive in the *S. officinarum* suspension culture at the log phase. We did not observe statistically significant changes to the proportion of living cells in the culture after exposure at 45 °C for 10 and 30 min (Fig. 1a, b). Exposure of the *S. officinarum* suspension culture at 50 °C activated a prolonged process of cell death, lasting all four days of the experiment. About 30–35 % of the cells in the culture remained alive by the end of this period (Fig. 1 a, b). It should be noted that the length of time the *S. officinarum* culture was treated at 50 °C for did not affect the intensity of the process of cell death (Fig. 1a, b). Exposure at 55 °C for 10 min caused an extensive process of cell death. About 30 % of cells remained alive in the culture immediately after the treatment (Fig. 1a). We did not observe any further cell death in the culture for the next two days. However, all the cells in the culture were dead after four days of treatment (Fig. 1a). Increasing exposure at 55 °C to 30 min turned out to be critical, as no more than 5 % of cells remained alive in the *S. officinarum* culture immediately after exposure (Fig. 1b). The effect of treatment at 60 °C (for 10 and 30 min) was similar, as only 3–5 % of the cells remained alive in the culture following treatment (Fig. 1a, b).

We can conclude that treatment at 45 °C was insufficient to cause irreversible damage in the *S. officinarum* cells, while the treatments at 55 and 60 °C were too extreme for the cells to form an adequate response. Treatment at 50 °C did not cause immediate cell death, however, the changes in cell metabolism were so significant that there was a process of cell death in the culture for four days following this treatment.

Changes in ROS levels and $\Delta\Psi_m$ in S. officinarum cells under the influence of high temperatures

All the high temperatures tested caused a significant increase in ROS levels in the *S. officinarum* cells (Fig. 2a, b). It was found that increasing the exposure time from 10 to 30 min

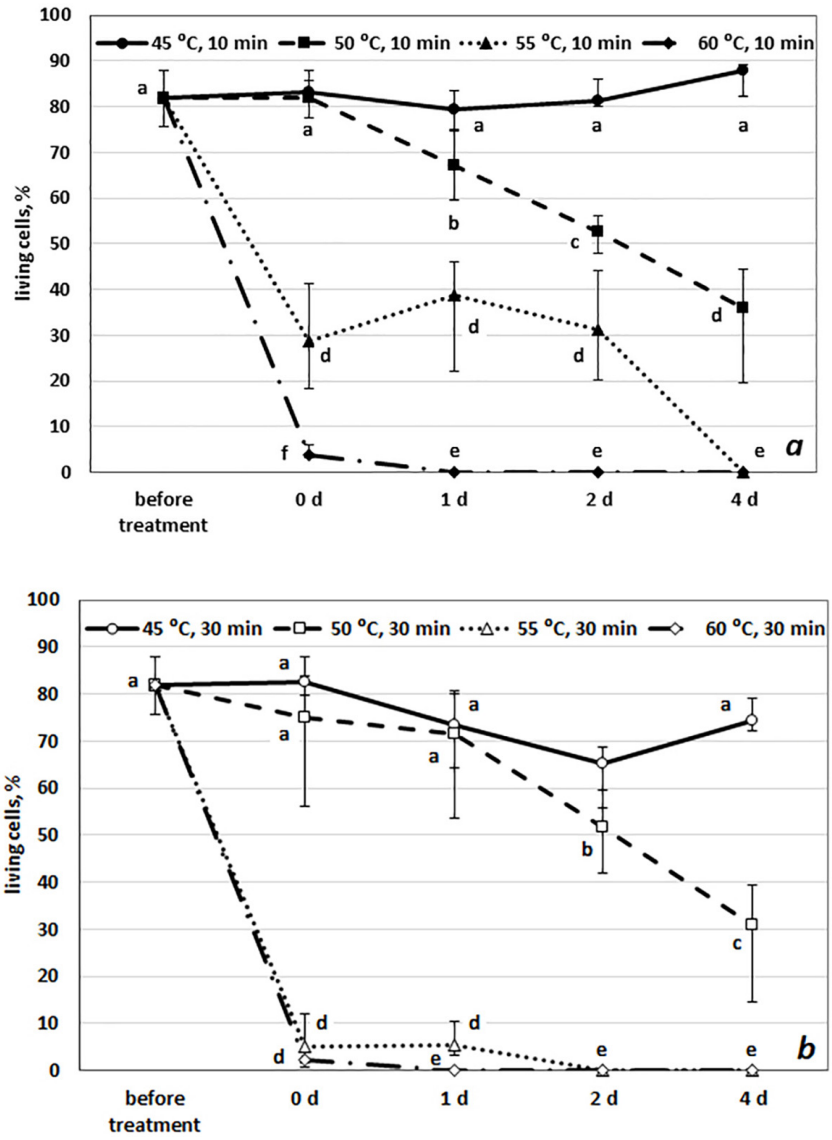

Fig. 1. Cell death processes in the *S. officinarum* suspension culture induced by high temperatures treatments for 10 (*a*) or 30 min (*b*). The figure shows the median (Q_{50}) and the error bars are the Q_{25} and Q_{75} percentiles (n = 5, $p < 0.05$)

at 45 and 50 °C leads to a further increase in ROS production in the cells (Fig. 2a, b). We detected a four-fold increase in DCF fluorescence intensity in the cells under all high temperature conditions. Increasing the exposure time from 10 to 30 min at 55 °C did not increase the ROS production in the cells (Fig. 2a, b). The treatment of the cell culture at 60 °C for 30 min led to a decrease in DCF fluorescence intensity, compared with the treatment of the culture at 60 °C for 10 min. It should be noted that ROS levels in the cells after the most severe heat exposure (60 °C at 30 min) did not significantly differ from those in the control cells (Fig. 2a, b).

The study of $\Delta\Psi_m$ using the JC-1 fluorescent probe showed that there were mitochondria with both high and low $\Delta\Psi_m$ in the control cells. This was proved by the fluorescence of JC-1 monomers

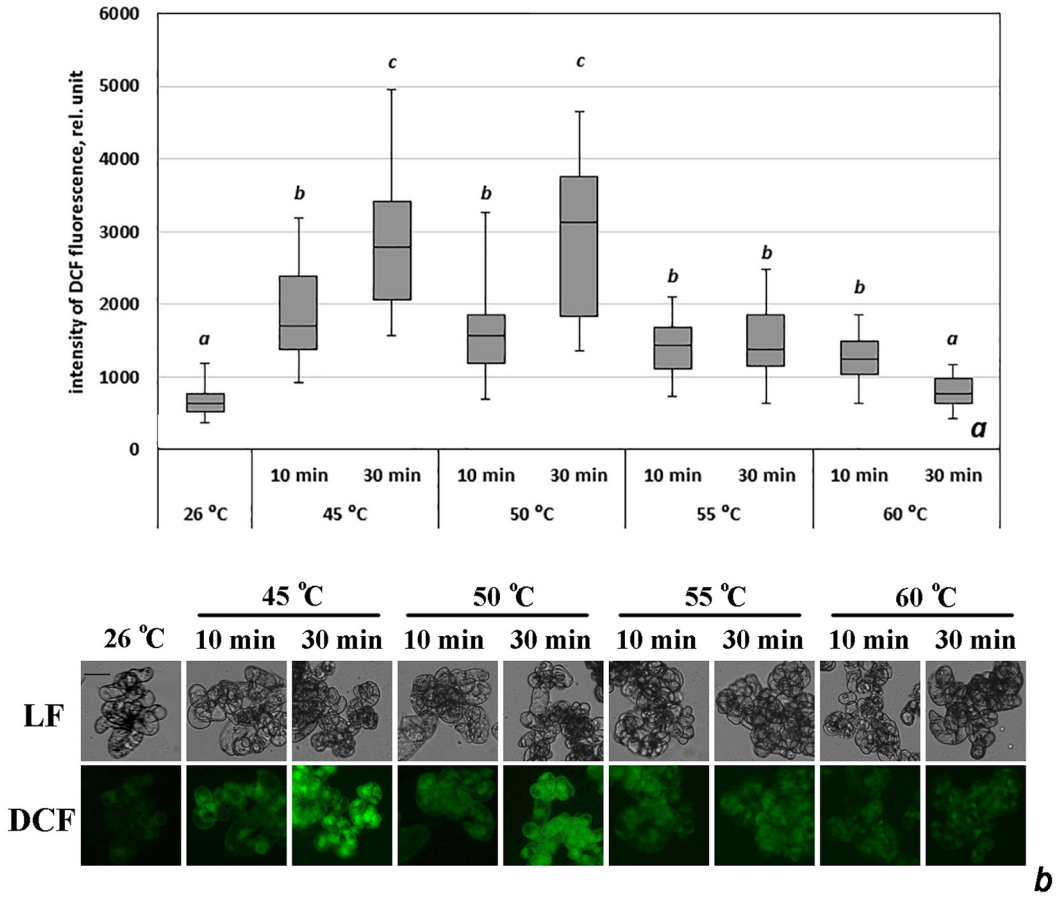

Fig. 2. ROS levels in the cells of the *S. officinarum* suspension culture under high temperature treatments: *a* – data counting (n = 5, $p < 0.05$); *b* – microphotograph of the *S. officinarum* cells. The line in the «box» is Q_{50}, the borders of the «box» limit the interquartile range between Q_{25} and Q_{75} and the «whiskers» present maximum and minimum values; rel. unit – relative units; 26 °C – control conditions; 45, 50, 55 and 60 °C – treatment temperature; 10 and 30 min – treatment exposure, LF – light field; DCF – 2,7-dichlorodihydrofluorescein diacetate, 1 μM; bar 50 μm

in the green spectral region and J-aggregates in the red spectral region (Fig. 3b). A statistically significant increase in the red fluorescence in the cells occurred only under short-term high temperature treatments for 10 min (Fig. 3a, b). Prolonged high temperature influence caused depolarisation of the inner mitochondrial membrane and almost complete disappearance of JC-1 red fluorescence in the *S. officinarum* cells (Fig. 3a, b). There was no red fluorescence and therefore an $\Delta\Psi_m$ increase in the cells of the *S. officinarum* suspension culture that had been exposed at 60 °C (Fig. 3a, b).

ROS production in S. officinarum suspension culture cells under high temperature conditions is caused by changes in mitochondrial activity

In order to determine how the ROS increase caused by high temperature treatments in *S. officinarum* cells is associated with mitochondrial activity, we used the carbonyl cyanide m-chlorophenyl hydrazine (CCCP, 20 μM). This compound is a protonophore and it uncouples oxidative phosphorylation in mitochondria. As a result, $\Delta\Psi_m$ on the inner mitochondrial membrane decreases. In this

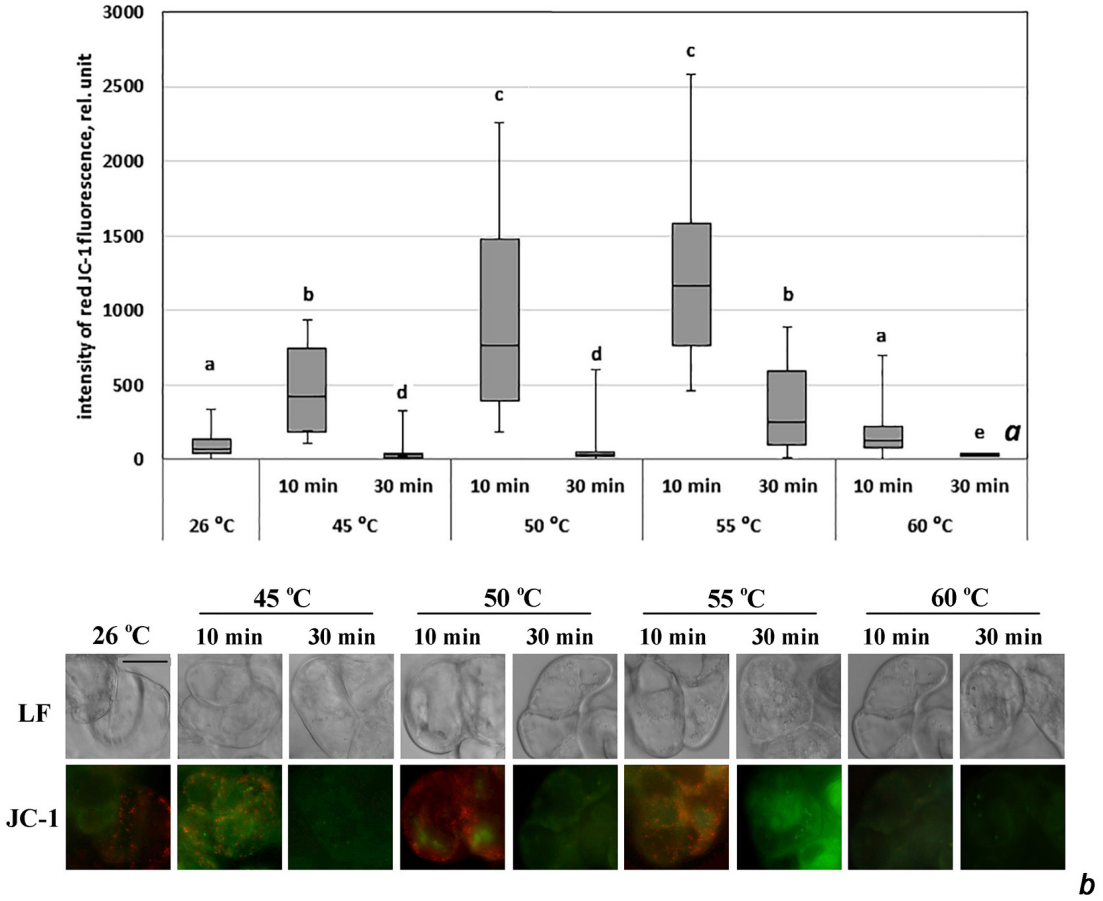

Fig. 3. $\Delta\Psi_m$ in cells of the *S. officinarum* suspension culture under high temperature treatments: *a* – red fluorescence data counting (n = 5, $p < 0.05$); *b* – microphotograph of the *S. officinarum* cells with green and red fluorescence. JC-1–5.5'.6.6'-tetrachloro-1.1'.2.2'-tetraethylbenzimidazolo-carbocyanine, 20 μM. Other designations correspond to Fig. 2

experiment, we only gave the high temperature treatment for 10 min (at 45, 50 and 55 °C), which caused both $\Delta\Psi_m$ and ROS content in the *S. officinarum* cells to increase. It was shown that the addition of CCCP to the culture during exposure to high temperatures prevented hyperpolarisation of the inner mitochondrial membrane, and red fluorescence of the J-aggregates was not observed (Fig. 4). To study the effect of CCCP on the ROS levels in the cells, it was added during the high temperature treatments, together with a DCF fluorescent probe. It was found that the ROS levels in *S. officinarum* cells were statistically significantly lower when the cells were simultaneously treated with high temperatures and CCCP, compared with only being given the high temperature treatment (Fig. 4). We can conclude that ROS production in *S. officinarum* cells under high temperature conditions was partially caused by changes in mitochondrial activity.

Changes to respiratory activity and AP contribution in S. officinarum suspension culture cells under high temperature treatments

The *S. officinarum* culture had a high respiratory rate under the control conditions

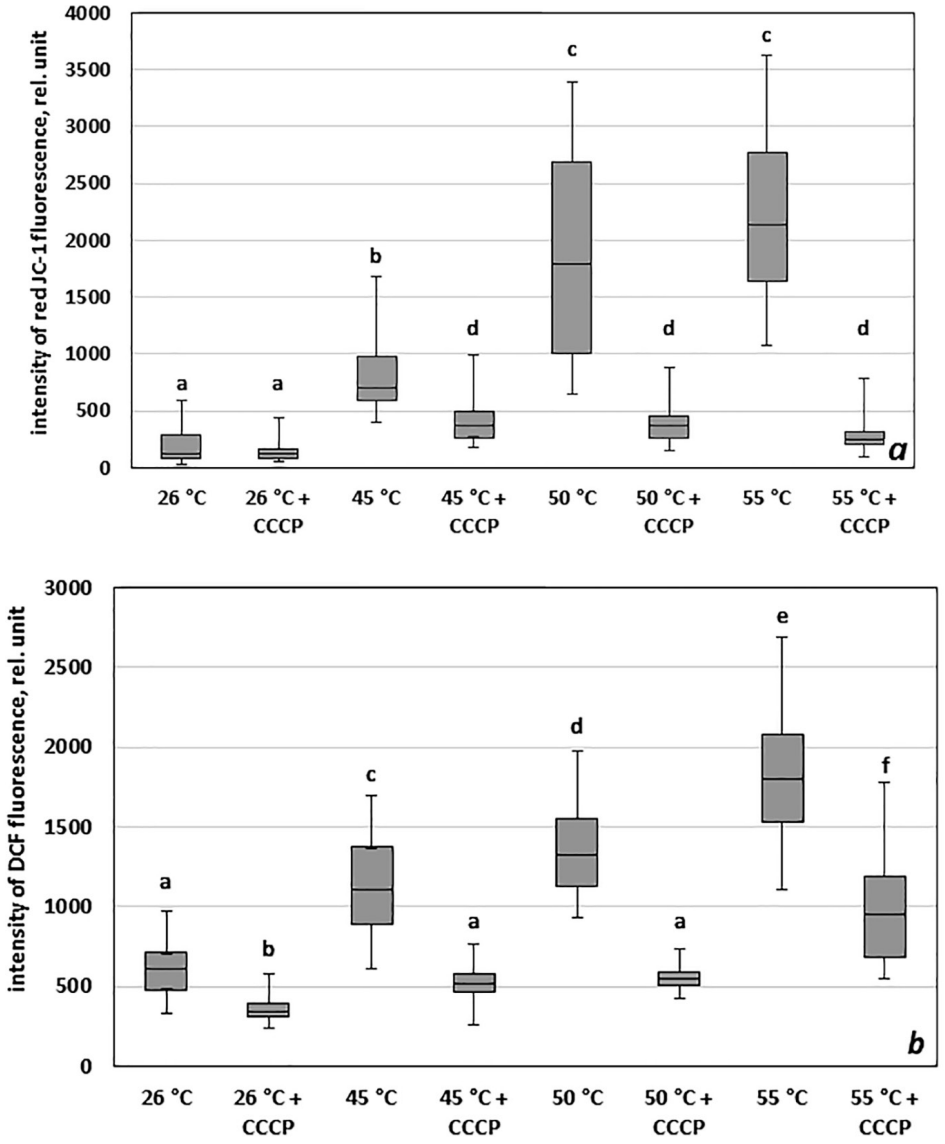

Fig. 4. $\Delta\Psi_m$ (*a*) and the ROS level (*b*) in cells of the *S. officinarum* suspension culture during exposure to high temperatures for 10 min with the addition of carbonyl cyanide m-chlorophenyl hydrazine (CCCP). 26 °C – control conditions; 45, 50 and 55 °C – temperature treatments without CCCP addition; 45 °C+CCCP, 50 °C+CCCP and 55 °C+CCCP – temperature treatments with CCCP addition, 5 μM. The line in the «box» is Q_{50}, the borders of the «box» limit the interquartile range between Q_{25} and Q_{75}, and the «whiskers» present maximum and minimum values (n = 3–5, $p < 0.05$); rel.unit – relative units

(about 3000 nmol O_2/(g FW × min) (Fig. 5a). The contribution of AP to the respiratory rate of *S. officinarum* cells was very significant. At the beginning of the experiment it was about 30 %, increasing to 40 % after one day (Fig. 5a, b). All the high temperature treatments resulted in a statistically significant reduction in the respiratory rate of the culture immediately following exposure (Fig. 5a). This reduction was mainly due to a decrease in CP activity (Fig. 5a). We observed a significant decrease in respiratory electron transport via AP only under the most severe high temperature exposure (55 °C for 30 min and 60 °C for 10 min, Fig. 5a). Treatment

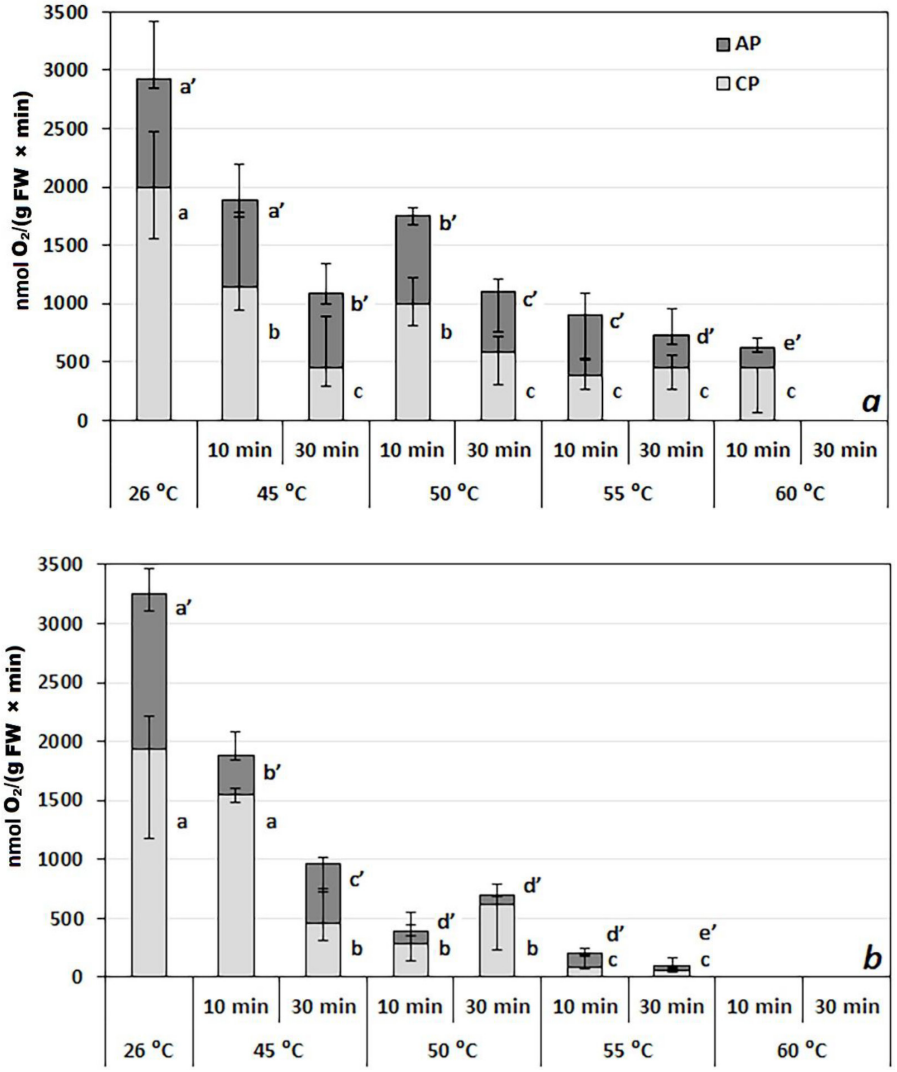

Fig. 5. Cyanide-sensitive (CP) and cyanide-resistant (AP) respiration in cells of the *S. officinarum* suspension culture after the high temperature treatments: *a* – immediately after the treatments; *b* – one day after the treatments. 26 °C – control conditions, 45, 50, 55 and 60 °C – temperature treatments; 10 and 30 min – treatment exposure. The figure shows the median (Q_{50}) and the error bars are the Q_{25} and Q_{75} percentiles (n = 5–10, $p < 0.05$)

of the culture at 60 °C for 30 min led to almost complete cell death and it was not possible to register respiration.

We obtained an interesting result analysing the respiratory rate of a *S. officinarum* culture treated at 45 °C. This exposure (for 10 and 30 min) did not cause cell death (Fig. 1a, b). Nevertheless, we observed a stable decrease in the respiratory activity of the cells throughout the experiment. It was 1.5 times lower than the control after 10 minutes of treatment (Fig. 5a, b). A 30-minute exposure had a more serious effect on cell respiration. The respiratory rate decreased by three times both immediately and after one day following treatment at 45 °C (Fig. 5a, b). It should also be noted that there was a predominant decrease in AP contribution to cell respiratory activity one day after treatment at 45 °C for 10 min up to about 18 % (Fig. 5b).

The changes caused by treatment at 50 °C to the respiratory activity of *S. officinarum* cells were also quite remarkable. This treatment had already led to a more significant decrease in the respiratory rate of *S. officinarum* cells than treatment at 45 °C, at one day post-treatment (Fig. 5b). The contributions of AP to respiration were also lower than the control at the corresponding period (29 and 11 % after 10 and 30 min of treatment, respectively, Fig. 5b). Particular attention should be paid to the fact that the decrease in respiration after exposure at 50 °C occurred faster than the process of cell death in the culture. Therefore, an interruption in respiratory activity preceded cell death (Fig. 1a, b and Fig. 5a, b). Treatments of the *S. officinarum* culture at 55 and 60 °C were so severe that they led to a complete interruption of cell respiration after only one day post-exposure (Fig. 5b).

It can be concluded that high temperatures influenced the respiratory rate of *S. officinarum* cells, even in the cases where we did not notice any negative effects on survival following treatment. Respiratory activity was critical for the formation of a cellular response to high temperature stress, and AP activity was the most stable under stress conditions.

Discussion

S. officinarum is an important agricultural plant belonging to the cereal or bluegrass family (Gramineae or Roaceae). Its region of origin is considered to be New Guinea (Daniels, Daniels, 1993). *S. officinarum* is a typical tropical plant and it has a sufficiently high level of resistance to high temperatures. Its optimum growth temperature is 30 °C. Due to this, we used temperatures that significantly exceeded the optimum to create high-temperature stress.

An increase in ROS in cells under the influence of high temperatures is one of the common responses of plants to stress. It has been shown that heat shock leads to an increase in ROS production in *Arabidopsis*, tobacco and winter wheat cells (Vacca et al., 2004; Zhang et al., 2009; Fedyaeva et al., 2014). In our earlier studies, we had also observed an increase in the formation of ROS in *S. officinarum* cells during short-term heat exposure (Lyubushkina et al., 2014b). It should be noted that ROS are products of normal plant metabolism and serve to regulate a wide range of processes, including growth and development. They also participate in the formation of plant responses to external influences (Katano et al., 2018). However, if unbalanced by scavenging processes, an increase in ROS production can cause irreversible damage to cells and lead to their death. In this study, we observed an increase in ROS production in all cases of heat treatment, except for at 60 °C for 30 min (Fig. 2a, b). In the latter case, the stress factor was so acute that all the cells had already died during exposure (Fig. 1b). Although other temperatures intensified ROS formation in *S. officinarum* cells, they did not always lead to cell death. We observed an increase in ROS levels after heat exposure at 45 °C, but cell death did not occur, either at the time of exposure or during the next four days (Fig. 1a, b and Fig. 2a, b). A similar increase in ROS production in *S. officinarum* cells was observed after heat exposure at 50 °C, and there was a process of prolonged cell death in the culture for the four days following treatment. Treatment at 55 °C also caused an increase in ROS levels in the cells, but the cell death caused at this temperature was much greater and occurred in a shorter period of time (Fig. 1a, b and Fig. 2a, b). Accordingly, we can conclude that under stress, ROS can trigger completely different response patterns in plant cells. Since implementing both adaptive mechanisms and PCD are energy consuming processes (Bras et al., 2005), it can be assumed that the realisation of these patterns depends on

the degree of mitochondrial damage by stress factors and their functional activity.

Fedyaeva et al. (2014) demonstrated the dependence of ROS levels in winter wheat cells on oxidative phosphorylation coupling under heat shock. CCCP prevented the hyperpolarisation of the inner mitochondrial membrane as well as ROS production (Fedyaeva et al., 2014). In our study, CCCP led to similar results (Fig. 4a, b). It should be noted that a decrease in DCF fluorescence intensity in the presence of CCCP does not occur at the control level, since processes associated with ROS production are not only present in mitochondria, but also in endoplasmic reticulum, peroxisomes and cytosol in a plant heterotrophic cell under stress (Katano et al., 2018).

The question is what changes in the function of plant mitochondria lead to subsequent cell death due to a critical increase in ROS levels? Our results suggest that it is important for the neighbouring cell to maintain respiratory intensity for the first day following stress exposure (Fig. 5b). Exposure at 45 °C (for 10 and 30 min) did not lead to cell death (Fig. 1a, b). The rate of cell respiration did not fall below 1000 nmol O_2/(g FW × min) in these cases (Fig. 5b). CP contribution increased to 90 % one day after treatment at 45 °C for 10 min (Fig. 5b). It can be assumed that the cells successfully scavenged increased ROS levels, and the ROS served as signalling molecules for cell reprogramming, triggering synthesis of the corresponding compounds necessary for acclimatisation (Kohli et al., 2019).

Treatment at 50 °C caused a prolonged process of cell death in the *S. officinarum* culture, and we observed a decrease in respiratory activity. However, this temperature did not significantly affect AOX activity, as AP contribution fell by 11 % compared with the control one day after the 30-min exposure (Fig. 5a, b). It is likely that this stability of AOX activity was necessary for the realisation of the PCD program in the culture. Borovik and Grabelnych (2018) showed that AP contribution increased in green winter wheat plants under the influence of high temperatures. This caused ROS levels to reduce in the mitochondria and chloroplasts (Borovik, Grabelnych, 2018). Our results are consistent with data obtained by Robson and Vanlerberghe (2002) using AS8 transgenic tobacco culture. They showed that the role of AOX in cell susceptibility to PCD is its ability to permanently weaken mitochondrial ROS production, preventing oxidative damage to cells. Regarding the highest temperatures that caused extensive cell death (55 and 60 °C), cell damage (including the mitochondria) was so great that the respiratory rate had already dramatically decreased during the treatment. Following this, there was an extensive and uncontrolled process of cell death (Fig. 1a, b; Fig. 5a, b).

Conclusion

In conclusion, while a plant cell under high temperature stress is able to provide for its increased energy needs and at the same time effectively limit the increase in ROS production, nothing threatens it. In this case the role of ROS is limited by the regulation of metabolism and serves to benefit the cell, providing a balanced formation of acclimation reactions. However, as soon as this balance shifts towards excessive ROS production, the respiratory ETC is destroyed, which entails subsequent disturbances of the entire cellular metabolism and ultimately leads to cell death.

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Some Aspects of the Relationship between Redox Metabolism and the Structure of Calciphytes

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Abstract. Calciphyte species form a systematically and structurally heterogeneous group of plants capable of tolerating highly stressful conditions. Various structural adaptations occur in calciphytes to protect them against excess light (leaf pubescence) and moisture loss (waxy coating). Their shoot structure determines the volume of primary plant production. The present work studied the relationship between the antioxidant status and structural features of some calciphyte species. Redox metabolism in plant leaves was assessed using parameters such as water content, photosynthetic pigments, soluble carbohydrates, water-soluble phenolic compounds, water-soluble and membrane-bound proteins, and lipid peroxidation (LPO) level. The data obtained showed that the contents of the components regulating redox metabolism correlate both with each other and with the structural parameters of plants. In particular, the content of photosynthetic pigments in multi-species communities is lower in taller plants than in low-growing ones. The content of phenolic compounds and the level of LPO in calciphyte leaves are associated with the level of development of wax covering. The plants forming clumps and vigorous shoots exhibit increased LPO activity.

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Особенности взаимосвязи редокс-метаболизма и структуры растений кальцефитов

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Аннотация. Растения кальцефитных флор образуют систематически и структурно неоднородную группу растений, способных переносить высокострессовые условия. Кальцефиты реализуют различные структурные адаптации, противодействуя избытку света (опушение), снижая потери влаги (восковой налет). Структура их побегов определяет объем первичной продукции растений. Цель настоящей работы – изучить взаимосвязи антиоксидантного статуса со структурными особенностями некоторых представителей кальцефитной флоры. Редокс-метаболизм оценивали по оводненности листьев, содержанию фотосинтетических пигментов, углеводов, фенольных соединений, водорастворимых и мембранно-связанных белков, интенсивности накопления продуктов перекисного окисления липидов (ПОЛ). Полученные данные показали, что количественное содержание компонентов, регулирующих редокс-метаболизм, демонстрирует наличие корреляционных связей как между отдельными группами этих веществ, так и со структурными показателями растений. В частности, в многовидовых сообществах у более высоких растений содержание фотосинтетических пигментов ниже, чем у низкорослых. Содержание фенольных соединений и уровень ПОЛ в листьях кальцефитов связаны с уровнем развития воскового налета. Растения, формирующие куртины и мощные побеги, характеризуются повышенной активностью ПОЛ.

Ключевые слова: кальцефиты, редокс-метаболизм, пигменты, фенольные соединения, углеводы.

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Introduction

Calciphyte species form a systematically and structurally heterogeneous group of plants capable of tolerating highly stressful conditions (Tyler, 2003; Malysheva, Malakhovsky, 2011). Calciphytes grow in specific habitats: soils exhibit a high initial pH value, rockiness, and a special moisture regime (Escudero et al., 2015). In addition to specific soil conditions, the plants are constantly exposed to winds, high air temperatures, and excessive insolation. Plants of chalk slopes and carbonate outcrops, as a rule, have a xeromorphic structure with their characteristic external features such as pubescence and smaller leaves, which often develop a silvery sheen (Kurovsky, 2009). They are represented by different life-forms: dwarf shrubs, semi-shrubs, root and rhizome perennials, etc. Low-growing, creeping plants predominate among calciphytes. The main part of photosynthetic apparatus can be represented by many small stem-covering leaves or well-developed root leaf rosettes, or openwork leaf rosettes (Lambers, Oliveira, 2019).

Thus, the conditions for the growth of calciphytes are highly stressful, and, therefore, different structural adaptations occur in the plants.

The physiological adaptations of plants are based on alterations in cellular processes resulting in changes of quantitative and qualitative composition of compounds closely related to the main metabolic pathways including redox metabolism. At the same time, redox regulation of cellular processes is considered as one of the fundamental mechanisms regulating cell functional activity (Martinovich, Cherenkevich, 2008). Under adverse environmental conditions, plants experience oxidative stress due to the formation of an increased amount of highly reactive oxygen species (ROS) (Gupta et al., 2018). Lipid peroxidation (LPO) is one of the redox regulation factors of enzymatic systems (Hasanuzzaman et al., 2020).

Under abiotic stresses such as drought, salinity, heavy metals, waterlogging, extreme temperatures, oxygen deprivation, etc., the activity of free-radical processes is also regulated by non-enzymatic antioxidants. The low molecular weight organic antioxidants (such as ascorbate, glutathione, carotenoids, tocopherols, flavonoids, betaines, etc.) sometimes can be more effective as metabolism protectors from ROS than enzyme systems. Phenolic compounds are the most common among them (Hasanuzzaman et al., 2020). They are broad-range mediators of biotic relationships, plant metabolism regulators, and participants in responses to abiotic stresses (D'Amelia et al., 2018). Recently, sugars have been considered as direct antioxidants (Bolouri-Moghaddam et al., 2010). The amount, chemical structure, and mechanism of action of antioxidants can vary significantly across plant species, such as calciphytes (Hasanuzzaman et al., 2020).

The aim of this work was to study the relationship between the elements of antioxidant system and the structural features of some calciphile plant species.

Materials and methods

Plant material

The study was conducted on 13 species from 9 families: *Artemisia salsoloides* Willd, *Anthemis trotzkiana* Claus (Asteraceae); *Pimpinella titanophila* Woronow, *Bupleurum falcatum* L. (Apiaceae); *Kraschenninnikovia ceratoides* (L.) Gueldenst. (Chenopodiaceae); *Onosma volgensi*s Dobrocz (Boraginaceae); *Gypsophila volgensis* Krasnova (Caryophyllaceae); *Astragalus zingeri*

Korsh., *Hedysarum grandiflorum* Pall. (Fabaceae); *Linum flavum* L., *L. uralense* Juz. (Linaceae); *Polygala sibirica* L. (Polygalaceae); *Reseda lutea* L. (Resedaceae). The plants were growing in the Shigonsky District of the Samara Region in two ecotopes: Ecotope 1–53°29′ N, 49°00′ E, and Ecotope 2–53°35′ N, 48°51′ E.

We took into account such structural parameters of the species as the height of plants (average values in cm), the vigor and the position of their shoots, the presence/absence of clumps and rosettes, and the comparative levels of leaf pubescence and wax coating (Ryabinina, Knyazev, 2009; Maevsky, 2014).

For biochemical analyzes, we used freshly cut leaves collected from 12–15 plants. Three independent biological samples, 0.2–2.0 g of wet weight, were prepared from the total mass and stored in liquid nitrogen until laboratory studies.

The water content of the tissues was calculated after determining the wet and air-dry weight and expressed as %.

Biochemical analyses

The lipid peroxidation rate in leaves of plants was determined by measuring the accumulation of malonyldialdehyde (MDA), determined using the color reaction with thiobarbituric acid at λ = 532 nm (Lukatkin, Golovanova, 1988).

The content of photosynthetic pigments was determined spectrophotometrically in an acetone extract (90 %) at λ = 662, 645, and 470 nm. The concentration of chlorophylls *a*, *b* and carotenoids was calculated using the method of H. K. Lichtenthaler (1987).

The water-soluble phenolic substances were extracted from air-dry plant material, their content was determined in aqueous extracts using the Folin-Chocalteu reagent according to the method (Swain, Hillis, 1959) in the drop modification proposed by the manufacturer of the Pancreac Quimica reagent, Spain. The absorbance of the solutions was determined on a photocolorimeter («KFK-2», Russia) using a red light filter at λ = 725–730 nm. Gallic acid was used to make a calibration graph.

The amounts of water-soluble proteins and membrane-bound proteins were determined by the O. N. Lowry method (Lowry et al., 1951) on a spectrophotometer (PromEcoLab PE-3000, Russia) at λ = 750 nm, using calibration curves with a standard solution of bovine serum albumin (Calbiochem, Germany) in distilled water and in a 0.05 % Triton solution X-100, respectively.

The content of soluble carbohydrates was determined in freeze-dried material. The weighed portion (0.3 g) was extracted by 70 % ethanol; the evaporated hydroalcoholic extract of the plant sample was dissolved in water and subjected to purification by solid-phase extraction method in cartridges with sorbents Disorb-60-S16T and Diasorb-60-Amine. After the purification, the solution was analyzed under the following conditions: column Kromasil 4.6 x 250 mm 100–5NH2 was used, the rate of eluent flow was 0.94 mL min^{-1}, and a refractometer served as a detector. The retention time of control substances served as the criterion of peak identification. Quantitative analysis was performed by absolute calibration method using peak areas as a reference.

Statistical analysis

The results of biochemical analyzes were presented as the average of three biological and three analytical replicates. The data obtained were presented in the form of arithmetic means (M), and the scattering of values was presented as standard errors (± SE). Comparison of quantitative data was carried out using one-way analysis of variance (One-way ANOVA) ($p < 0.05$) followed by the Tukey's test to compare the means. Calculations were performed using the Statistica 6.0 for Windows and Past 3 software.

For an expert assessment, the species were assigned conditional scoring characteristics, which were entered into the data matrix and, together with biochemical indicators, were used to calculate the pair correlation coefficients in the Microsoft Excel (Zaitsev, 1984).

Results and discussion

The typical structural and morphological traits of the aboveground organs of the species studied in this work are presented in Table 1.

According to the literature data, plants differ in both habit and characteristics of leaves. Low (20–40 cm) and medium-sized (50–60 cm) plants predominate among the species studied here, but one species, the subshrub *K. ceratoides*, forms vigorous leafy shoots up to 1 m in height and more. Most of the plants have shoots rising above the surface of the soil substrate. In four species, the main apparatus of photosynthesis is well-developed near-root leaf rosettes, and two species have openwork leaf rosettes. The other species are characterized by a relatively weak development of the leaf surface; they form thin branched shoots. The species also differ significantly in the pubescence of leaves and stems and the thickness of the wax coating.

Redox metabolism in plant leaves was assessed using parameters such as water content, photosynthetic pigments, soluble carbohydrates, water-soluble phenolic compounds, water-soluble and membrane-bound proteins, and MDA level (Table 2). The content of MDA, one of the final products of lipid oxidation, varied within wide limits depending on the plant species (0.1–1.7 mmol g^{-1} dry weight). Calciphytes grow in water-limited soil conditions, and, therefore, it is important for them to maintain water homeostasis. Our results showed that the water content of the leaves was 51.1–78.4 % of dry weight, which corresponds to the values of xerophytic plants (Table 2). The largest water deficit was found in *O. volgensis*, *K. ceratoides* and ***A. zingeri*** plants. The water content in their leaves was 1.2 times lower than in most of the species studied.

The content of photosynthetic pigments in leaves is considered as an indicator of the physiological state of plants, which characterizes the efficiency of the photosynthetic apparatus. The concentration of chlorophylls varied from 2.4 to 6.3 mg g^{-1} and carotenoids from 0.4 to 1.2 mg g^{-1} of dry weight. Plants *L. flavum*, *P. sibirica*, and *R. lutea* had high pigment contents. The calciphytes studied in the present work were heliophytes. They exhibited a high chlorophylls *a*/*b* ratio. This parameter varied in the range of 2.4–4.1.

The carbohydrates synthesized by plants in the photosynthesis process are the initial material for plastic and energy metabolism. In addition, they are known to have antioxidant properties and the ability to maintain the water balance of cells (Zakhozhiy et al., 2019). The total content of soluble carbohydrates in the dry mass of leaves varied from 14.9 to 78.0 mg g^{-1} (Table 2). Most of the calciphyte species demonstrated the predominance of disaccharides. The lowest concentration of sugars was found in the leaves of *H. grandiflorum*, which was mainly due to the low content of disaccharides. These plant species are able to slow down or speed up metabolic processes depending on external conditions (Ilyina, 2019). One can assume that the low sugar content compared to other species is a consequence of these processes.

The evaluation of protein components showed that the amount of membrane-bound proteins ranged from 6.8 to 81.0 mg g^{-1} and water-soluble ones – from 12.9 to 267.6 mg g^{-1} dry weight. Moreover, the content of water-soluble proteins may be 1.2–8.0 times greater than the content of the membrane-bound ones. The largest amount of membrane-bound proteins was found in the leaves of *B. falcatum*,

Table1. Life-forms and morphometric parameters of calciphytes

Species	Life-form	Shoot height, cm	Vigor of shoots	Position of shoots relative to the surface of the substrate	Formation of dense aerial shoots*	The presence of a root leaf outlet*	The degree of leaf pubescence**	The degree of the waxy coating of leaves**
A. trotzkiana	SS	Up to 25 cm	Medium	CP	+	+(openwork)	++	-
A. salsoloides	SS	Up to 50 cm	High	RP	+	-	-	++
A. zingeri	SS	Up to 50cm	Medium	CP	+	+	+++	-
B. falcatum	PP	Up to 60 cm	Low	RP	-	+(openwork)	-	+
G. volgensis	PP	Up to 90 cm	Low	RP	-	-	-	+
H. grandiflorum	PP	Up to 50 cm	Medium	RP	+	+	++	-
K. ceratoides	SS	1 m and more	High	RP	+	-	++	-
L. flavum	PP	Up to 40 cm	Medium	RP	+	-	-	+
L. uralense	PP	Up to 20 cm	Low	RP	+	+	-	+
O. volgensis	PP	Up to 50 cm	High	CP	+	-	+++	-
P. titanophila	SS	Up to 40 cm	Low	RP	-	+	++	-
P. sibirica	PP	Up to 20 cm	Low	RP	+	-	+	-
R. lutea	PP	Up to 60 cm	High	RP	-	-	-	+

Note: SS – semi-shrubs; PP – perennial plants; CP – creeping plants; RP – raised plants. Expert characteristics criteria for some morphological features of plants: *absence (–) or presence (+); **absence (–); minimal (+), medium (++), maximal (+++) levels.

Table 2. Physiological and biochemical parameters of calciphyte leaves

Species	MDA, mmol g^{-1} dry weigh	Water content, %	Total chlorophylls	Carotenoids	Chlorophyll a/*b*	Monosaccharides	Disaccharides	Water-soluble proteins	Membrane-bound proteins	Phenolic compound
			mg g^{-1} dry weight							
Ecotope 1										
A. salsoloides	1.7±0.5a	64.8±1.4bc	2.8±0.2d	0.6±0.1c	2.5	40.4±2.1a	19.3±0.5c	267.6±11.3a	37.5±3.4c	132.5±7.0a
A. trotzkiana	0.2±0.1b	70.1±2.3b	2.4±0.3d	0.4±0.0d	2.4	32.5±2.6b	40.2±2.5a	84.9±4.5e	14.7±2.0e	25.9±2.3e
P. titanophila	0.4±0.1b	64.8±2.0b	3.5±0.4c	0.7±0.0c	2.9	29.7±1.3b	23.1±2.0b	144.6±12.5c	22.7±1.9d	60.0±3.0b
K. ceratoides	0.2±0.1b	57.7±1.0d	3.8±0.1c	0.7±0.0c	3.8	7.6±1.7d	11.6±1.5e	153.7±12.4c	42.1±1.4c	48.4±2.1c
O. volgensis	0.3±0.1b	51.1±1.1e	3.8±0.2c	0.6±0.1c	2.8	14.0±1.3c	24.6±1.3b	69.5±1.9f	10.2±2.0f	37.3±3.0d
G. volgensis	0.1±0.0c	67.5±2.7b	2.7±0.1d	0.5±0.1cd	3.2	1.2±0.1f	25.0±1.4b	80.3±1.8e	15.1±1.0e	26.4±1.0e
L. uralense	0.3±0.1b	66.0±1.6b	3.5±0.3c	0.6±0.1c	2.9	–	–	12.9±1.2h	6.8±1.0g	–
Ecotope 2										
A. zingeri	0.4±0.1b	57.6±2.0d	4.4±0.3b	0.9±0.1b	3.0	4.8±0.3d	15.6±1.0d	122.9±12.0d	17.7±1.1e	47.6±2.2c
B. falcatum	0.4±0.1b	62.6±3.0bc	4.6±0.3b	0.9±0.1b	4.1	–	–	184.0±15.6b	81.0±3.0a	51.9±2.5c
H. grandiflorum	0.5±0.2b	62.0±2.1c	4.9±0.3b	0.9±0.1b	3.1	5.0±1.0d	9.9±0.9e	120.0±11.0d	15.0±1.5e	26.5±2.5e
G. volgensis	0.1±0.0c	67.5±3.5b	2.7±0.2d	0.6±0.1c	2.9	3.4±0.3e	21.7±1.6b	112.3±9.0d	24.3±2.0d	57.0±3.6b
L. flavum	0.4±0.1b	78.2±4.0a	6.3±0.4a	0.9±0.2ab	2.9	32.1±2.0b	45.9±3.0a	81.2±2.5e	57.8±3.0b	27.5±2.1e
K. ceratoides	0.5±0.2b	53.1±2.0e	2.6±0.3d	0.6±0.1c	3.3	–	–	136.9±13.8cd	56.9±3.6b	17.5±2.0f
P. sibirica	0.1±0.0c	62.4±2.4c	5.4±0.5b	1.2±0.1a	2.9	–	–	44.1±1.2g	34.3±6.0c	27.4±2.6e
R. lutea	0.4±0.2b	78.4±3.0a	5.1±0.3b	0.8±0.1bc	2.9	38.5±2.1a	26.6±2.3b	115.7±10.4d	17.6±2.3e	54.9±2.4b

Note: data are mean ± SE ($n = 9$). Different letters indicate significant differences at the level of $p < 0.05$.

and water-soluble proteins were the highest in *A. salsoloides*. It is well-known that individual components of water-soluble proteins are responsible for protection against oxidative stress (Orlova et al., 2007).

Plant polyphenols have pronounced antioxidant and antiradical properties. Due to the antioxidant effect, phenolic compounds with the combined action of the antioxidant system are able to «quench» free radicals, stabilize and protect cell membranes from damage, prevent the autolysis of lysosomes, mitochondria, etc. (Martinovich, Cherenkevich, 2008; Gupta et al., 2018; Zagoskina, Nazarenko, 2016). The content of phenolic compounds, as well as proteins and carbohydrates, which are also involved in the redox metabolism, varied widely across the plant species (17.5–132.5 mg g^{-1} dry weight). In two species used as an example – *K. ceratoides* and *G. volgensis* – the content of phenolic compounds depends not only on the species traits but also on the growing conditions. Thus, in the leaves of both species, sampled in different ecotopes, the content of phenolic compounds differed by more than a factor of two.

The content analysis of primary and secondary metabolism components, including those involved in the regulation of redox metabolism, shows that their amounts vary significantly in different calciphyte species.

We used the correlation analysis method to identify the possible relationship between some structural traits and physiological and biochemical parameters. To this purpose, the primary expert assessment of structural features was fulfilled. The plants were assigned quantitative estimates: heights (average values in cm), the relative degree of development of aerial shoots (from 1 to 10 points), the presence or absence of clumps (0–1 points), the presence of outlets (absence – openwork rosettes – well-developed rosettes, from 1 to 3 points), the presence and degree of development of pubescence (from 0 to 3 points), the presence and degree of development of wax coating (from 0 to 2 points), the position of the shoots (creeping over the substrate or rising above it (1 and 2 points). Table 3 presents the results of the analysis of relationships between the metabolic parameters and structural traits of the calciphytes studied. Both positive and negative correlations were found.

A high positive pair correlation was found between photosynthetic pigment contents – chlorophyll *a*/chlorophyll *b*, chlorophyll *a*/carotenoids, chlorophyll *b*/carotenoids ($r = 0.91$; 0.86; 0.78, respectively). Similarly, a high positive correlation was found between the MDA level/the content of water-soluble proteins, the MDA level/the content of phenolic compounds ($r = 0.77$ and 0.79, respectively). That means that under stressful conditions, all antioxidant components are activated, contributing to the development of adaptive plant responses. The accumulation of water-soluble phenolic compounds in leaves of calciphytes correlated with the level of development of wax coating on the vegetative parts of the shoots ($r = 0.67$), and an average positive level of correlation between the development of wax coating and MDA level ($r = 0.52$) was also revealed.

It is well-known that under water deficient conditions, wax coating on the surface of plant leaves not only contributes to the regulation of the water balance, but also, unlike pubescence, better reflects sunlight, thereby protecting the assimilation organs of plants from ultraviolet radiation and burns (Lambers, Oliveira, 2019).

The plant height expressed in conditional points negatively correlated with the content of photosynthetic pigments (r values from –0.36 to –0.59), which is quite consistent with the level of light exposure of plants with different shoot heights in multispecies communities – the maximal for the highest and more limited for the

Table 3. Paired correlation coefficients of biochemical and structural parameters of calciphytes

Parameters	Dry mass	Monosac-charides	Disac-charides	Phenolic compounds	Chlorophyll *a*	Chlorophyll *b*	Carotenoids	Water-soluble proteins	Membrane-bound proteins	MDA
Dry mass	1.00									
Monosaccharides	**-0.55***	1.00								
Disaccharides	**-0.64****	**0.54***	1.00							
Phenolic compounds	-0.08	**0.44**	**-0.28**	1.00						
Chlorophyll *a*	-0.25	0.14	0.11	-0.20	1.00					
Chlorophyll *b*	**-0.36**	**0.37**	0.25	-0.07	**0.91*****	1.00				
Carotenoids	-0.03	-0.06	-0.23	-0.13	**0.86*****	**0.78*****	1.00			
Water-soluble proteins	0.12	**0.35**	**-0.44**	**0.82*****	-0.15	-0.20	-0.07	1.00		
Membrane-bound proteins	0.03	0.31	0.28	0.05	**0.33**	0.09	**0.33**	**0.46**	1.00	
MDA	0.01	**0.53***	0.15	**0.79*****	-0.11	0.05	-0.09	**0.77*****	0.23	1.00
Plant height	0.14	**-0.54***	-0.32	-0.12	**-0.40**	**-0.59***	**-0.36**	**0.34**	**0.31**	-0.11
Vigor of shoots	**0.32**	0.26	-0.23	0.19	-0.24	-0.13	**-0.35**	0.12	-0.25	**0.40**
Clumps	**0.32**	0.12	**-0.36**	0.13	-0.18	-0.09	-0.30	0.11	**-0.36**	**0.36**
Rosettes	-0.09	**-0.45**	-0.11	-0.22	0.14	0.08	0.16	0.03	-0.10	-0.15
Leaf pubescence	**0.57***	-0.25	-0.24	-0.27	-0.01	0.04	0.03	-0.12	**-0.39**	-0.15
Waxy coating of leaves	**-0.38**	0.26	0.09	**0.67****	-0.08	-0.03	-0.07	**0.36**	0.28	**0.52***
Shoot position	**-0.52***	0.31	0.17	0.05	-0.06	-0.11	-0.05	0.13	**0.31**	0.07

Note: The average level of correlation ($|0.3 < r < 0.6|$) is indicated by bold font and light gray color; high ($|r > 0.6|$) – by bold font and dark gray; *The reliability of the correlation coefficient at a confidence level of 0.95 for a sample size of 15 pairs of values (species) corresponds to the values of the correlation coefficient of 0.51 and higher; **At a confidence level of 0.99 – the correlation coefficient is 0.63 and higher; ***At a confidence level of 0.999 – the correlation coefficient of 0.75 and higher.

undersized. At the level of an average positive relationship, an increase in lipid peroxidation was observed in plants that had more vigorous shoots and formed clumps of shoots (r = 0.40 and 0.36, respectively), which is logically associated with the successful development of species that form a significant amount of phytomass of densely leaved shoots in ecologically harsh habitats.

Multiple connections of medium strength, both positive and negative, were found for a number of monosaccharides as a group of primary metabolites. The level of their accumulation was associated with the activity of metabolic processes (water soluble proteins) and tissue antioxidant status (MDA). There was a tendency towards a decrease in the number of monosaccharides in plants with an increase in their height (r = –0.54) or the formation of basal rosettes (r = –0.45).

Conclusion

The data obtained showed that the quantitative level of the elements of the redox system correlates both with each other and with the structural parameters of calciphytes. In particular, the content of photosynthetic pigments in multi-species communities in taller plants is lower than in low-growing ones. The content of phenolic compounds and the level of MDA in calciphyte leaves is associated with the level of development of wax covering. In ecologically harsh habitats, plants forming clumps and vigorous shoots are characterized by increased LPO activity.

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Impact of Microorganism Priming on Oxidative Processes and the Antioxidant Defense System of Grapes Infected with Downy Mildew

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Abstract. Priming plants with natural agents, including microorganisms, is a promising alternative to chemical methods of protection in growing plants and, particularly, in viticulture. However, the molecular mechanisms of the priming phenomenon are still not fully elucidated. The antioxidant system and reactive oxygen species are known to effectively modulate plant responses to various external influences. This study aimed to identify the relationship between the priming of grapes by microorganisms and the functioning of the antioxidant system in a protective response to downy mildew infection. The experiment was carried out on leaf discs of Muscat blanc susceptible to downy mildew infected with *Plasmopara viticola* and treated with microorganisms incompatible with the pathogen, as well as with the corresponding symbiotic microorganisms. During the compatible interaction between *P. viticola* and grapes, oxidative processes were suppressed with viniferin formation. Leaf treatments with *Saccharomyces cerevisiae* and *Bacillus subtilis* effectively curbed the development of downy mildew on grape leaves. Priming with these microorganisms did not lead to a significant change in the biochemical parameters of grapes. Nevertheless, subsequent downy mildew infection initiated the formation of viniferin and maintained H_2O_2 content at a high level. Thus, priming with microorganisms eliminates the physiological effects of compatible interactions between downy mildew and grapes associated with blocking oxidative processes. To suppress pathogen development, host defenses and antagonistic effects of microbial priming agents are required.

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Keywords: *Plasmopara viticola*, *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Venturia inaequalis*, compatible interaction, antioxidant system.

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Окислительные процессы и антиоксидантные реакции у винограда при заражении милдью на фоне прайминга микроорганизмами

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Аннотация. Прайминг растений естественными природными агентами, в том числе микроорганизмами, представляет интересную альтернативу химическим методам защиты в растениеводстве и в виноградарстве в частности. Однако молекулярные механизмы этого явления до конца не ясны. Известно, что антиоксидантная система растений и образование активных форм кислорода эффективно модулируют реакции растений на множество внешних воздействий, в том числе биотический стресс. В связи с этим цель исследования – выявление связи прайминга винограда микроорганизмами и функционирования элементов антиоксидантной системы в защитном ответе на заражение милдью. Эксперимент проводили на листовых дисках винограда восприимчивого к милдью сорта Мускат белый, зараженных *Plasmopara viticola* на фоне обработок несовместимыми и симбиотическими организмами. Определение содержания стильбенов, аскорбиновой кислоты проводили методом капиллярного электрофореза, содержание перекиси водорода, ТБК-активных веществ и активность ферментов – спектрофотометрически. Совместимое взаимодействие между *P. viticola* и виноградом характеризовалось подавлением окислительных процессов и образованием микроботоксичной окисленной формы ресвератрола – виниферина. Эффективными в сдерживании развития милдью были обработки листьев *Saccharomyces cerevisiae* и *Bacillus subtilis*. Прайминг данными микроорганизмами не приводил к существенному изменению биохимических показателей винограда, однако последующее заражение инициировало образование виниферина и сохранение содержания H_2O_2 на высоком уровне. Таким образом, прайминг микроорганизмами нивелирует физиологические эффекты совместимого взаимодействия милдью и винограда, связанные с блокированием окислительных процессов у винограда. Для полного сдерживания развития патогена требуются защитные реакции растения-хозяина и антагонистическое воздействие со стороны микроорганизмов – агентов прайминга.

Ключевые слова: *Plasmopara viticola*, *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Venturia inaequalis*, совместимое взаимодействие, антиоксидантная система.

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Introduction

Most cultivated grape varieties are affected by fungal pathogens, which have harmful effects on all photosynthetic organs, disrupt nutritional processes – and in such a way disrupt the accumulation of plastic substances ensuring plant wintering – and damage both berries and the planting material harvested from the affected plants. This unavoidably reduces the crop yield, inflicting large economic losses and making the issue of devising novel methods to protect viticulture especially pressing. A correlation between the susceptibility of *Vitis vinifera* L. to *Plasmopara viticola* and the lack of an effective pathogen recognition system in these species was proposed; namely, their defense system is not sufficiently efficient to fully activate and successfully curb the growth of pathogens (Di Gaspero et al., 2007). In addition, several studies have indicated some mechanism leading to the temporary activation of protective genes and proteins (Milli et al., 2012; Figueiredo et al., 2017). However, this system is neither fast nor sufficiently reliable to prevent the spread of the pathogen.

Viticulture largely depends on fungicides, the widespread use of which makes it possible to obtain high-quality grapes but at the same time leads to several problems: significant environmental load, decreased dietary properties of grapes, and considerable financial costs. Moreover, this may also be one of the reasons for pathogen resistance. As a viable alternative to chemical pesticides, immunity inducers may be used, including salicylic acid and its analogs (Dufour et al., 2013), jasmonic acid and methyl jasmonate (Belhadj et al., 2006), synthetic resistance inducers (Wang et al., 2019), and various ethylene producers (Chong et al., 2008). Although these methods are effective (Van Hulten et al., 2006), they are difficult to apply in the field, since the reactions caused by inducers of plant immunity are strictly determined by the time of their impact: the cycles of pathogen development and their spread in agrocenosis may start when the plastic and energy resources of the plant have already been exhausted. In this case, the simultaneous occurrence of many biotic and abiotic stresses tends to be the key problem. At present, the mechanism of setting plant defensive priorities remains understudied; therefore, the prediction of their metabolic properties is problematic, let alone generating a reliable defense response.

Under natural conditions, the immune response of plants is triggered either by substances secreted by the pathogen or the products of the plant's own cell destruction. Natural competitors of pathogens effectively reduce the spread of diseases in agricultural plants. Considering these two facts, the systems for biocontrol of pathogens exploiting antagonistic or sensitizing (incompatible) organisms are gaining an increasing renown. Antagonistic organisms make

it possible to effectively control the number of pathogens, as they induce the death of the latter. The priming effect is achieved by harnessing so-called «sensitizing» organisms, which activate long-term systemic immune responses in plants. These responses do not require any serious energetic and plastic rearrangements of plant metabolism and are preferable in the long run. The interaction of a plant with nonspecialized pathogens can provoke an immune response, i. e., to sensitize a plant, although it hardly ever leads to disease (Yacoub et al., 2016; Mutawila et al., 2017; Baccari et al., 2019). Thus, by the time of a subsequent interaction with a specialized pathogen able to suppress the host's immune responses (Nascimento et al., 2019), the plant will have generated a pool of metabolites and protective substances to reduce the spread of a compatible pathogen.

Most environmental stresses, including biotic stresses, lead to the production of reactive oxygen species (ROS) in plants, which cause oxidative stress (Miller et al., 2007). At low concentrations, ROS play vital physiological roles in plants: maintaining a normal concentration of H_2O_2, which takes part in the biosynthesis of the cell wall and phytoalexins (Jabs et al., 1997), and providing protection against pathogens (Lamb, Dixon, 1997). At higher concentrations, ROS can damage membranes, proteins, chlorophyll, and nucleic acids (Scandalios, 1997), which may be dangerous for plants since biotic stresses are known to cause an increase in the production of reactive oxygen species (ROS) in plant cells (Tang et al., 2010). Hence, inducing the plant defense system against natural pathogens may be mediated by the antioxidant system involved in oxidative stress. This system relies on a class of antioxidant enzymes that maintain the levels of ROS produced in large amounts after the pathogen infects the plant in subtle equilibrium (De Gara et al., 2003). The aim of the present study was to investigate the relationship between the priming phenomenon in grapes and the functioning of the elements of the antioxidant system in a protective response to downy mildew infection.

Materials and methods

The experiment was carried out on leaf discs of Muscat blanc, the grape species susceptible to downy mildew. The leaves were collected from plants grown in growing chambers. The photoperiod was 16/8 h. The experiment was carried out on leaf discs in Petri dishes with wet filter paper. The following treatments were exploited: *Bacillus subtilis* (Bs), *Saccharomyces cerevisiae* (Sc), *Venturia inaequalis* (Vi), and control – water. The Sc treatment was carried out by spraying the leaf discs with a suspension at a concentration of 10^6 cells mL^{-1} from a pure culture of *S. cerevisiae* obtained from the leaves of the Merlot grape variety. To prepare the Vi inoculum, 2 g *V. inaequalis* mycelium (provided by the Laboratory of biotechnological control of phytopathogens and phytophages of the North Caucasian Federal Scientific Center of Horticulture, Viticulture, Wine-making) was used per 20 mL distilled water. The final Vi suspension was sprayed onto the leaf discs. The Bs treatment was carried out using a commercial preparation of *B. subtilis* strain 26 D (Fitosporin). Fitosporin powder (0.2 g) was diluted in 100 mL water, and after 2 h, the grape leaf discs were sprayed according to the manufacturer's instructions. Twenty-four hours after treatment, a spore suspension of *P. viticola* (Pv) 3×10^6 spores mL^{-1} was applied by spraying. The spore suspension was prepared by washing off sporangia from the symptomatic leaves of greenhouse grape plants previously infected with downy mildew.

The effect of priming on physiological changes in grape leaves was assessed 48 and 96 hpi (hours post inoculation). The effect of downy mildew infection on biochemical

changes in the grape leaves was determined 24 and 72 h after infection and appeared to be equal at 48 and 96 hpi. Downy mildew development was assessed by the proportion of leaf disc area yielding sporangiophores at 72 h after the infection.

To determine the total content of stilbenes, an extract was prepared by incubating 0.1 g of a sample triturated in liquid nitrogen overnight in the dark at +4 °C in 2 mL 95 % ethanol. The next day, the extract was centrifuged at 15000×g for 15 min; the supernatant was used for further analyses of the content of stilbenes. To determine the ascorbic acid level, an extract from leaves was prepared by incubating 0.1 g of the sample overnight at +4 °C in 10 % ethanol. Then, the stilbenes were determined by capillary electrophoresis on a Kapel 105 (Lumex, Russia); the focus of interest was resveratrol, viniferin, piceid and ascorbic acid in the cell extract. The enzymatic activity of superoxide dismutase (SOD), guaiacol peroxidase (POX) and lipid peroxidation (content of thiobarbituric acid reactive substances (TBARS)) were determined specrophotometrically using a Unico2800 UV/VIS spectrophotometer (USA) (Radyukina et al., 2012). The hydrogen peroxide content was determined by the FOX1 method (Wolff, 1994).

Results

B. subtilis is an antagonistic microorganism used in crop production to protect against various groups of pathogens. Sc (wine yeast) are natural symbionts of grapes that populate berries and vegetative organs. Vi was chosen as a priming agent as it is an incompatible fungal pathogen for grapes. The importance of microorganisms in controlling the development of downy mildew on the grape leaf discs varied. Vi failed to demonstrate effectiveness in reducing downy mildew development on grape leaves. Bs treatment reduced the leaf sporulation area by 23 %; Sc treatment showed the greatest efficacy (Fig. 1).

At 48 hpi, the content of hydrogen peroxide in grape leaves decreased after Vi and Sc treatments, as well as because of Pv infection. During this period, the H_2O_2 content did not change in the Bs treatment. Pv infection against a background of priming with microorganisms led to H_2O_2 content recovery to the control values at 48 hpi. At 96 hpi, H_2O_2 content in the Pv and Sc

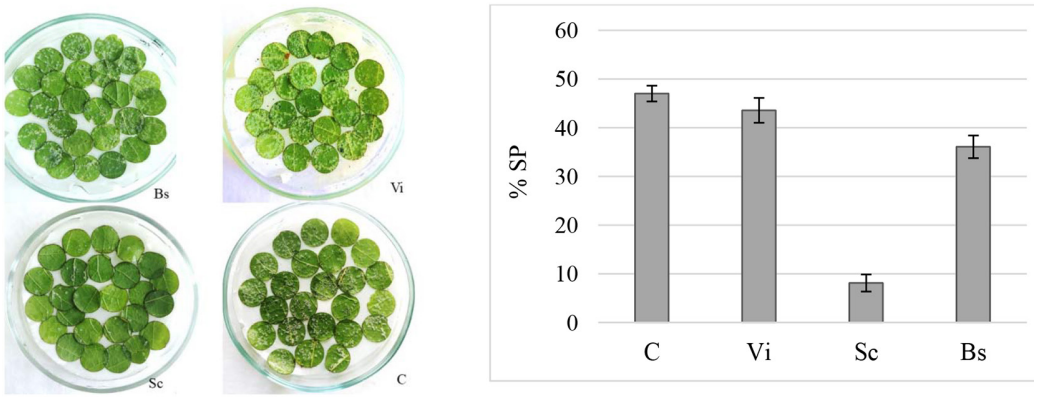

Fig. 1. Downy mildew development on grape leaf discs with experimental treatments (a). Leaf disc area yielding sporangiophores (b). The figures indicate the means with standard errors (n = 50). SP – percentage of the leaf disc area yielding sporangiophores, C – mock inoculated, Bs –*Bacillus subtilis* treatment, Sc – *Saccharomyces cerevisiae* treatment, and Vi – *Venturia inaequalis* treatment.

treatments was below the control and similar to each other. The hydrogen peroxide content in the Bs treatment at 96 hpi increased in comparison with that at 48 hpi. Downy mildew infection with microbial priming resulted in an increase in H_2O_2 content compared with the uninfected treatments. The content of hydrogen peroxide decreased in leaves with Bs + Pv, similar to that at 48 hpi and 96 hpi (Fig. 2).

The content of TBARS during Sc treatment decreased compared with that of the control at 48 hpi. Bs increased the TBARS content, as did Vi + Pv infection. Interestingly, Bs + Pv and Sc + Pv treatments reduced or did not change TBARS content compared with non-Pv-infected variants. At 96 hpi, Pv reduced the TBARS content in grape leaves, while Vi and Bs increased the TBARS content in the leaves. Interestingly, in Vi + Pv, Bs + Pv, and Sc + Pv treatments, the TBARS content decreased compared with the uninfected Pv variants (Fig. 2).

P. viticola significantly reduced the activity of superoxide dismutase at 48 hpi. In contrast, Vi, Sc, and Bs increased the enzyme activity. *P. viticola* infection against a background of microorganism treatments also had a stimulating effect on superoxide dismutase activity. At 96 hpi, the activity of superoxide dismutase in Pv increased significantly, while in other treatments with microorganisms, it decreased and matched the control values. At the same time, Pv infection against a background of priming with microorganisms increased SOD activity. Interestingly, at 48 and 96 hpi in the Sc + Pv treatment, SOD activation was less intense than in the other two variants of infection against a background of priming (Fig. 3a).

At 48 hpi, peroxidase activity increased intensively during Pv infection; however, treatment with Vi, Sc, Bs, or combinations of Pv with other microorganisms did not significantly increase the activity of peroxidases. At 96 hpi, peroxidase activity under Pv infection was high, while Bs significantly increased POX activity (Fig. 3b). In Vi and Sc treatments, the POX activity increased compared with the control, whereas it was lower than that in Pv and Bs treatments at 48 hpi. The POX activity during Pv infection against a background of priming was lower than that in Pv infection in mock-inoculated leaves at 96 hpi but increased compared with that at 48 hpi (Fig. 3b).

The content of ascorbic acid in grape leaves increased at 48 hpi under Pv infection, Sc treatment, and Bs inoculation (Fig. 4a). Pv infection against a background of priming with Bs and Sc led to a decrease in the content of ascorbate. At 48 hpi, the content of ascorbic acid

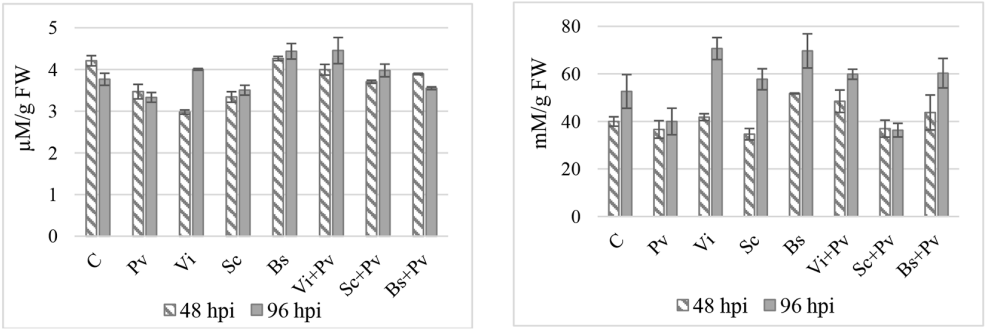

Fig. 2. The contents of hydrogen peroxide (a) and TBARS (b) in grape leaves. The figures represent the means with standard errors (n = 3). FW – fresh weight.

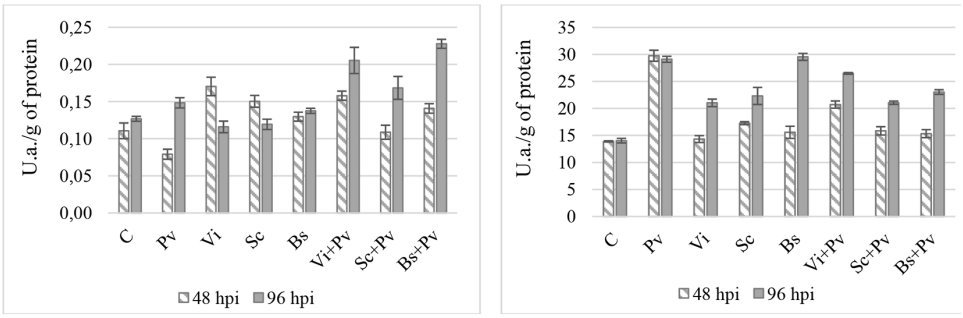

Fig. 3. The activity of antioxidant enzymes in grape leaves: (a) superoxide dismutase (SOD) and (b) guaiacol peroxidase (POX). The figures represent the means with standard errors (n = 3). U.a. – units of enzyme activity

in grape leaves in Vi and Vi + Pv treatments was maintained at the control level or slightly higher. At 96 hpi, the same trend continued: the ascorbate content was high in Pv, Sc, and Bs treatments and decreased upon Pv infection against a background of priming. In Vi and Vi + Pv treatments, as well as at 48 hpi, it was at the control level or slightly higher (Fig. 4a).

The content of resveratrol in grape leaves at 48 hpi was higher in all variants of the experiment than in the control (Fig. 4b). However, the resveratrol content appeared to be lower in the variant Pv, Vi, and Sc + Pv treatments. At 96 hpi, a pronounced increase in the content of resveratrol in the Sc treatment and a decrease in combinations of Pv with priming microorganisms were observed (Fig. 4b).

Viniferin content increased significantly at 48 hpi in Sc, Bs, Sc + Pv and Bs + Pv treatments (Fig. 4c). In the Pv treatment, it was slightly higher than at the control level. In the Vi treatment, both without infection and with Pv infection, the viniferin content did not change compared with the control. At 96 hpi, a decrease in the content of viniferin in the experimental variants was marked. In the Pv treatment, as at 48 hpi, the content of viniferin in the leaves did not differ significantly from the control level. Pv infection against a background of Sc and Bs led to the restoration of the viniferin content to the control level (Fig. 4c).

At 48 hpi, the piceid content increased in Pv, Vi, and Bs treatments (Fig. 4d). In Vi + Pv and Bs + Pv treatments, the piceid content decreased slightly and remained at the control level. The content of piceid in the Sc treatment remained at the control level, and with an additional infection with Pv decreased below the control. At 96 hpi, the same tendency of changes in the content of piceid in grape leaves was noted as at 48 hpi. Generally, the highest piceid content was observed in the Vi and Bs treatments, and the lowest was observed in the Sc treatment (Fig. 4d).

Discussion

P. viticola, an obligate parasite of grapes, can suppress the defense reactions of the host plant by synthesizing molecules that disrupt the defense mechanisms of grapes. The susceptibility of *V. vinifera* to *P. viticola* tends to be caused by the lack of an effective pathogen recognition system that would allow full activation of the defense system to successfully curb the growth of pathogens. *P. viticola* reduced the content of hydrogen peroxide in grape leaves compared with the control samples at 48 hpi and 96 hpi. At 48 hpi, a decrease in SOD activity and a major increase in the activity of POX, as well as an increase in

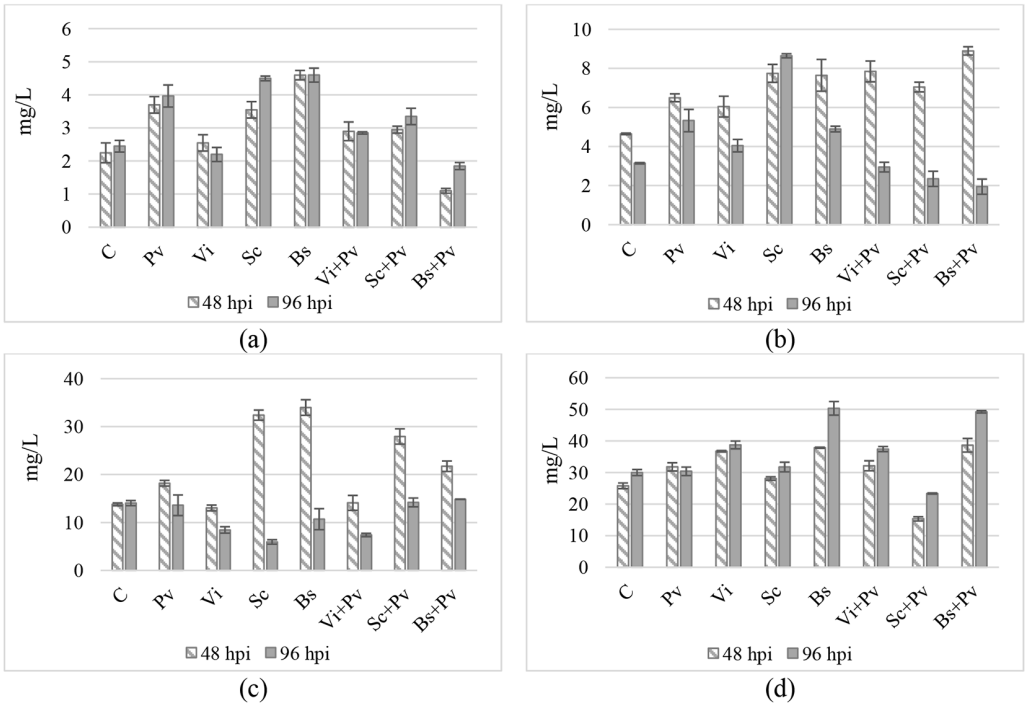

Fig. 4. The contents of ascorbic acid (a), resveratrol (b), viniferin (c) and piceid (d) in grape leaves. The data indicate the means ± standard errors (n = 3)

the content of ascorbate, were observed. These changes conditioned a decrease in the content of H_2O_2, while lipid peroxidation remained the same: such an environment is required for a compatible biotrophic pathogen to infect the plant (Polesani et al., 2008; Nascimento-Gavioli et al., 2017). Other peroxidases were also observed at elevated levels (up to a 20-fold increase) in a compatible interaction between susceptible Pinot Noir and *P. viticola* (Milli et al., 2012), which may have been caused by a compatible interaction between Pv and *V. vinifera*. The cells of the host plant, influenced by a biotrophic pathogen, reduce the formation of ROS and, to a lesser extent, contribute to oxidative stress (Nascimento et al., 2019).

Stilbenes play a special role in protecting grapes from various diseases (Pezet et al., 2004), as they, especially resveratrol, generally exhibit high antioxidant activity (Biais et al., 2017). Resveratrol is also glycosylated in the form of piceid, which is protected from enzymatic oxidation (Regev-Shoshani et al., 2003). Resveratrol and piceid have little or no toxic activity against *P. viticola*, while viniferin is highly toxic and can be considered an important marker of grape resistance to downy mildew (Pezet et al., 2004). Viniferins are products of the oxidation of resveratrol by peroxidases (Ros Barcelo et al., 2003). A slight increase in the content of viniferin, resveratrol, and piceid observed at 48 hpi indicates the activation of defensive processes, as well as an insufficient level of oxidative processes that typically activates the synthesis of toxic viniferin.

V. inaequalis treatment appeared to be ineffective in controlling Pv. The production of H_2O_2 decreased relative to the control at 48 hpi. Infection with Pv against a background of Vi restored the content of hydrogen peroxide to

the control level at 48 hpi, while it was above the control at 96 hpi. Thus, oxidative processes during Pv infection against a background of Vi seemed to be very pronounced, while SOD activity increased, and POX activity was lower than that by Pv infection alone. No changes in the contents of ascorbic acid and viniferin were observed, while resveratrol and piceid increased compared with the control. Supposedly, Vi is able to neutralize some effects resulting from the compatible interaction of grapes with Pv. However, to curb the growth of Pv, it is necessary to «correct the direction» of oxidative processes toward the conversion of phenolic compounds. In addition, a low accumulation of H_2O_2 with its compatible interaction with a biotrophic pathogen may be insufficiently toxic for the pathogen and, therefore, may be unable to inhibit the growth of the pathogen (Figueiredo et al., 2017; Nascimento et al., 2019).

The content of hydrogen peroxide, TBARS, and SOD activity increased in the Bs treatment at 48 hpi, which was more effective in controlling downy mildew development. Notably, the degradation of ascorbate occurred most intensively in the Bs treatment after Pv infection in the experimental variants. Infection of plants with pathogens leads to lipid peroxidation (Patel, Williamson, 2016), i. e., the cell membrane is disrupted, and cellular integrity is destroyed, which leads to further ROS formation. The lack of change in antioxidant capacity may be the cause of lipid peroxidation or oxidative processes in susceptible cultivars. At the same time, these processes could be considered an attempt of a susceptible cultivar to activate the first line of defense reactions using a burst of ROS (Mandal et al., 2011; Nascimento et al., 2019). Importantly, Bs significantly increased the formation of viniferin above the control values. Thus, with Bs priming, the effect of compatible interaction between grapes and Pv decreases, while the formation of a toxic oxidized form of resveratrol (viniferin) increases; however, the latter reaction does not suffice to suppress the development of Pv.

The most effective Sc treatment showed similar changes brought to Pv infection in lipid peroxidation rates. The SOD activity was higher than that of the control, and at 96 hpi, it increased significantly in the Sc+Pv treatment, which led to an increase in the H_2O_2 content. The POX activity in infected and uninfected Sc leaves did not change. Ascorbate degradation in the Sc+Pv treatment, in contrast to the Bs+Pv treatment, was less intense. The decrease in piceid and resveratrol content and the corresponding significant increase in viniferin content during Pv infection against a background of Sc indicate «effective» oxidative processes inducing the formation of viniferin.

Conclusion

This study revealed that *B. subtilis* and *S. cerevisieae* priming, which reduced the development of *P. viticola* on grape leaves, ensures a higher level of H_2O_2 and maintains the associated SOD activity, as well as inhibiting the growth of peroxidase activity in the susceptible Muscat blanc grape variety, thus preventing a compatible interaction between *P. viticola* and *V. vinifera*. The experiment showed that lipid peroxidation did not have a significant effect on the control of downy mildew development. Therefore, a substrate for peroxidases can be assumed to play one of the key roles in the effective suppression of downy mildew development, since, with a decreased resveratrol concentration, a more pronounced formation of viniferin was observed with *S. cerevisiae*, while with less effective *B. subtilis*, intensive degradation of ascorbate occurred. Additional antagonistic action by priming agents might be needed to secure more effective containment of downy mildew development on susceptible grape varieties.

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