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Research Article

Metabolite profiling of leaves of three *Epilobium* species

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BACKGROUND: The ability of plants to adapt to oxygen deficiency is associated with the presence of various adaptations, many of which are mediated by significant changes of metabolism. These changes allow resistant wetland plants to grow even in oxygen-deficient environment.

AIM: The aim of the study was to carry out metabolic profiling of the leaves of the wetland species *Epilobium palustre* and *Epilobium hirsutum*, and the mesophyte species *Epilobium angustifolium* in order to identify the most characteristic metabolome traits of hypoxia-resistant plants.

MATERIALS AND METHODS: Metabolite profiling was performed by GC-MS. Statistical analysis of metabolomics data was processed using R 4.2.1 Funny-Looking Kid.

RESULTS: The resulting profile included about 360 compounds. 70 of these were identified and 50 compounds were determined to a class. Sugars (64) were the most widely represented in the obtained profiles. 16 amino and 20 carboxylic acids, lipids and secondary compounds have been identified. Significant differences were revealed between the profiles of leaf metabolomes of mesophyte *E. angustifolium* and hydrophytes *E. hirsutum* and *E. palustre*. The mesophyte was characterized by high levels of sugars. The metabolomes of wetland Epilobium species practically did not differ from each other and were characterized by the accumulation of amino acids, including GABA shunt intermediates, dicarboxylic acids of the Krebs cycle, and metabolites of glycolysis and lactic acid fermentation, which reflects the stimulation of anaerobic respiration, nitrogen metabolism, and alternative pathways of NAD(P)H reoxidation in wetland plants.

CONCLUSIONS: Traits of metabolic profiles detected in hydrophyte *Epilobium* species can be used to assess the degree of plant resistance to oxygen deficiency.

Keywords: hypoxia; hydrophytes; mesophyte; metabolomics; GC-MS; *Epilobium palustre*; *E. hirsutum*; *E. angustifolium*.

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Научная статья

Метаболическое профилирование листьев трех видов кипрея

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Способность растений адаптироваться к кислородной недостаточности связана с наличием различных приспособлений, многие из которых опосредованы существенными изменениями обмена веществ. Эти изменения позволяют устойчивым растениям-гидрофитам расти даже в дефицитной по кислороду среде. Цель настоящей работы состояла в метаболическом профилировании листьев гидрофитных видов *Epilobium palustre*, *Epilobium hirsutum* и мезофитного вида *Epilobium angustifolium* для выявления наиболее характерных изменений метаболома, свойственных устойчивым к дефициту кислорода растениям. Профилирование метаболитов проводили методом газовой хроматографии-масс-спектрометрии. Полученный профиль включал около 360 соединений. Из них было идентифицировано 70 и еще 50 соединений были определены до класса. В полученных профилях наиболее широко были представлены сахара (64) и их производные. Идентифицировано 16 аминокислот, 20 карбоновых кислот, а также липиды и вторичные соединения. Были выявлены существенные различия между профилями метаболомов листьев мезофитного *E. angustifolium* и гидрофитных *E. hirsutum* и *E. palustre*. Для мезофита были свойственны более высокие уровни сахаров. Метаболомы гидрофитных кипреев практически не отличались друг от друга и характеризовались аккумуляцией аминокислот, в том числе интермедиатов ГАМК-шунта, дикарбоновых кислот цикла Кребса и метаболитов гликолиза и молочнокислого брожения, что отражает стимуляцию у них анаэробного дыхания, азотного обмена и альтернативных путей реокисления НАД(Ф)Н.

Ключевые слова: гипоксия; гидрофиты; мезофит; метаболомика; ГХ-МС; *Epilobium palustre*; *E. hirsutum*; *E. angustifolium*.

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ЭКО•ВЕКТОР

BACKGROUND

Elucidating the mechanisms of plant adaptation to changing environmental conditions is one of the most important problems of modern plant biology. Plants are obligate aerobes; however, they can often grow in environments where oxygen is low (hypoxia) or completely absent (anoxia). Oxygen deficiency develops due to water excess during spring floods, heavy rainfall, and continuous waterlogging, affecting plant cultivation. According to the Food and Agriculture Organization of the United Nations, average world crop losses from hypoxia and anoxia ranged from 10% to 50% per year [1, 2]. Plants of natural ecosystems living in conditions of waterlogging or in the aquatic environment also also face the problem of oxygen deficiency..

Prolonged submergence hinders the diffusion of oxygen, carbon dioxide, and ethylene [3, 4] and leads to the accumulation of toxic gases and ions [4, 5]. These result in the suppression of aerobic respiration [6, 7] and photosynthesis [8]. Oxygen depletion causes several cellular consequences, including severe energy crisis, acidification of the cytoplasm, inhibition of protein synthesis, and accumulation of toxic products of anaerobic metabolism, particularly acetaldehyde and ethanol [4, 6, 9]. The production of reactive oxygen [10, 11] and nitrogen [2, 12] species is also triggered by anoxia and can cause oxidative damage to lipids and proteins [13–15]. Anaerobic conditions stimulate glycolysis, with lactic acid fermentation occurring in the early phase and causing cytoplasmic acidification [4, 6]. In the later phase, ethanol fermentation predominates [9, 16].

Adaptations to oxygen deficiency are most often studied in cultivated mesophyte or terrestrial plant models (Arabidopsis, tobacco), which are intolerant or middle tolerant to hypoxia. In contrast, rice is a typical aquatic plant or hydrophyte that can resist oxygen deficiency. Studies on the adaptive mechanisms of flora living in aquatic and bog ecosystems are few compared with studies on cultivated plants. Conventional classical biochemical methods have revealed that hydrophytes (e.g. rice, *Salix alba*, and *Glyceria maxima*) are characterized by low respiration rates and oxygen consumption compared with mesophytes (e.g. wheat, *Populus petrowskiana*, *Phaseolus vulgaris*) [6, 9]. The transition to anaerobic metabolim in adapted species occurs gradually, while in intolerant plants, a rapid, short-term increase in glycolytic activity can be observed in the first hours of anaerobic exposure [5, 6, 9]. Glycolysis requires an abundant supply of carbohydrates to proceed continuously, as it leads to overconsumption of sugars, especially in mesophytic species [6, 9]. Plants in aquatic and semi-aquatic habitats store significant amounts of carbohydrates in their rhizomes (up to 50% of their dry mass) and seeds, which are utilized during hypoxic or anoxic germination [4, 5, 7, 17]. However, despite the presence of carbohydrate reserves in intolerant plants, they are unable to mobilize their polysaccharide stores. This is due to the lack of expression of the genes encoding starch phosphorylase and α-amylase during anoxia. Stimulation of lactic acid and alcoholic fermentation occurs in intolerant mesophytic species during shorter periods of oxygen deficiency at increased levels than adapted hydrophytes [5, 6, 9]. The intermediate and final products of fermentation contribute to the cell toxicity (acetic aldehyde, ethanol, methylglyoxal) and acidification of the cytosol (lactate). Well-adapted species accumulate less alcohol and lactate during prolonged oxygen starvation due to the retardation of anaerobic metabolism and the excretion of end products into the flooded water and into the air through the aerenchyma [6, 7, 9]. Preventing the accumulation of toxic metabolites can also be achieved by activating metabolic pathways that produce compounds other than ethanol or lactate. Many wetland plants redirect glycolysis intermediates to the synthesis of alternative end products, such as malate, succinate, glycerol, alanine, and gamma-aminobutyric acid (GABA). These alternative metabolites in hypoxia can be used to re-oxidize reduced nicotinamide adenine dinucleotide (NADH) to NAD^+ required for glycolysis [6, 9]. Additionally, accumulation of alanine and GABA can prevent cytoplasmic acidosis during oxygen starvation [4, 17].

In recent years, several systems biology approaches (transcriptomic, proteomic, and metabolomic) have been used to study the mechanisms of hypoxia and anoxia tolerance. The metabolomic profiles of the thale cress (*Arabidopsis thaliana*) [18], *Lotus japonicus* [19], rice [20], soybean [21], wheat [22], and other cultivated plants have been reported. Significant modifications in the metabolism of sugars, organic acids of Krebs cycle, and amino acids have been identified, which agree with results using conventional methods of biochemistry.

In the literature, a few studies reported the changes in the metabolome during oxygen deficiency in hydrophytes. Anoxic exposure led to metabolomic shifts in both the roots and shoots of *Zostera marina* [23]. In the shoots, anoxia did not affect sugar content but increased lactate, pyruvate, succinate, and 2-oxoglutarate. Shoot levels of proline, glutamate, and glycine did not change, while alanine and GABA increased. In comparison, fructose and glucose contents were significantly reduced in the roots, while alanine, proline, and GABA content increased. There were trends of succinate and fumarate accumulation and malate consumption. Profiling the metabolites of *Potamogeton anguillanus* shoots under hypoxia and anoxia revealed the accumulation of soluble sugars and lactate. During hypoxia, malate, citrate, and succinate were slightly increased, an effect that was significantly accelerated under anoxic conditions. The same patterns were

demonstrated for alanine, proline, glutamate, glutamine, and GABA [24].

Metabolomic responses to oxygen deficiency have also been characterized by comparing tolerant and intolerant forms of rice, wheat, and soybean. In rice, the M202(Sub1) isogenic line, which carries the *SUB1A* gene on an *Oryza sativa* L. ssp. *japonica* (variety M202) genetic background, exhibited higher tolerance [25]. In oxygen deficiency, both M202(Sub1) and M202 varieties showed decreased sucrose and increased glucose levels, which were more pronounced in M202(Sub1). Krebs cycle metabolites, except citrate, were generally exhausted in both forms, while most amino acids, including GABA, were accumulated to a greater amount in M202(Sub1). These results suggest that the *SUB1A* gene enhances carbon and nitrogen metabolism involved in adaptations to hypoxia. Another comparative analysis was performed in two varieties of wheat, a plant that is much more sensitive to oxygen deficiency than rice. Varieties Frument (intolerant) and Jackson (tolerant) were used [22]. Changes in the carbohydrate content in the shoots did not reveal differences in flood tolerance as soluble sugars decreased to very low levels in the shoots of both varieties. Twelve of the seventeen amino acids analyzed (asparagine, glutamine, isoleucine, leucine, valine, lysine, methionine, phenylalanine, tyrosine, tryptophan, threonine, and proline) increased in both varieties, while five amino acids (alanine, aspartate, glutamate, serine, and glycine) did not change or decreased slightly. However, amino acid accumulation was higher in the shoots of the Frument variety, which could indicate significantly higher levels of protein degradation compared to the tolerant Jackson variety. Unfortunately, these data cannot reveal a clear relationship between the dynamics of amino acids and tolerance in different varieties.

A comparison of two soybean varieties, BR4 (tolerant) and Embrapa 45 (intolerant), was also performed [26]. In this previous study, both the shoot and root metabolomes of these varieties were compared under flooding conditions and revealed significant differences in their carbohydrate, carboxylate, and amino acid profiles. Soluble sugars accumulated markedly in the roots of the intolerant variety but these changes were absent in the leaves and roots of the tolerant one. In the roots, a larger decrease in malate and fumarate was noted in the tolerant variety, while a greater accumulation of acetate and succinate was seen in the intolerant variety. In the leaves, the levels of acetate and succinate decreased only in the tolerant variety (BR4). Alanine and GABA levels increased in the roots and decreased in the shoots. These metabolomic changes in the roots of the tolerant soybean variety overlapped with the profile seen in the seedlings of tolerant rice.

From these studies, we conceptualized a promising approach of comparing the metabolic profiles of closely related hydrophytes and mesophytes, which differ in tolerance to flooding and grow in different biotopes on the same territory. The data from this approach can help to identify metabolic changes that are typical of adapted plants and can serve as guide for monitoring of oxygen deficiency-tolerant varieties, which is of interest for plant breeding and agrobiotechnology.

Such plants include the fireweed genus (*Epilobium* L.) or the evening primrose family (*Onagraceae* Juss.). The genus includes up to 222 species [27] distributed on all continents except Antarctica [28]. Fireweeds typically grow in the subarctic, subantarctic, and temperate regions, while in the subtropics and tropics, their distribution is limited to cool mountain biomes. In European Russia, including the Leningrad region, there are 12 species of native and invasive fireweeds [29], some of which are aggressive adventives and are included in the regional Black Books [30]. Mesophytic fireweed (great willow herb) [*Epilobium angustifolium* L. = *Chamaenerion angustifolium* (L.) Scop.] is a perennial long-rhizome plant that grows in light forests, along skirts and glades, near roads, and on railway embankments. It is also one of the pioneer types of plants in the overgrowing of clearings and burnt areas, where it develops on light sandy soils. Great hairy willow herb (*Epilobium hirsutum* L.) and marsh willow herb (*E. palustre* L.) are hydrophytic (helophytic) species that live along the margins of swamps, banks of water bodies, swampy meadows, and damp ditches [29].

In the present study, we aimed to carry the metabolic profiling of leaves of two hydrophytic and one mesophytic fireweed species to identify metabolites characteristic of plants adapted to oxygen deficiency.

MATERIALS AND METHODS

Research objects

We used the leaves of two hydrophytic species of fireweed (*Epilobium palustre* L. and *E. hirsutum* L.) and one species mesophytic fireweed (willow herb) [*E. angustifolium* L. = *Chamaenerion angustifolium* (L.) Scop.] in the experiments. Plants were collected in the canyon of the River Lava (Kirovskiy district, Leningrad region; coordinates of the collection site: starting point 59°52'56.40''N, 31°35'6.09''E; endpoint 59°53'4.05''N, 31°35'5.70''E). The Lava River valley is covered with a broad-leaved forest of elm, ash, and maple growing on calcareous deposits of the Cambrian and Ordovician periods. The riverbed is rich in rifts and shallows, where numerous hydrophytic plants grow on stony limestones. The climate at the collection site is temperate, moderate continental, and humid, with an average temperature of –7.7°C in January and 17.7°C in July. The annual amount of precipitation is 500–900 mm. The duration of the growing season is 85 to 135 days [31].

For the study of hydrophytes, we harvested the leaves of plants that grew in a water reservoir or in its immediate vicinity, partially or completely submerged in water, indicating limited oxygen access to organs and tissues. Willow herb (*E. angustifolium*) leaves were collected nearby in a dry meadow outside the flood zone. We analyzed 3–4 biological replicates collected from different plants. Leaves of approximately the same size and one generation were selected. Inspection was performed to ensure no samples were affected by pathogens and phytophages. Samples were collected over two years in mid-July. Plant tissue (200 mg) was weighed on a portable electronic scale, cut into small pieces directly at the collection site, and fixed in 1 mL of methanol.

Sample preparation

Within 2–3 h, the samples were delivered to the laboratory, where the methanol extract was transferred into a new microtube, and the plant residues were ground in a bead mill (Tissue Lyser LT, QIAGEN, Germany, 50 beats/s, 3 times for 2 min) with 1 mL methanol. Extraction was performed for 1 h on a TS-100C thermoshaker (BioSan, Latvia) at 800 rpm, 4°C. After extraction, the samples were centrifuged for 10 min at 15,000 rpm, 4°C and then the residue was washed twice with 500 µL of methanol on a TS-100C thermoshaker, with centrifugation of the sample each time (10 min at 15,000 rpm, 4°C). The supernatants were combined and the total extract was evaporated to dryness in a Labconco CentriVap vacuum evaporator (USA). The air in the microtubes was replaced with gaseous nitrogen and placed in a freezer at –80°C for storage until analysis.

For analysis, dry material was dissolved in pyridine (100 µL) containing internal standard C_{23} (tricosane) and 100 µL of a silylating agent, 99% solution of bis(trimethylsilyl)-N, O-trifluoroacetamide with 1% trimethylchlorosilane (Sigma, USA). Samples were derivatized by incubation on a TS-100C thermoshaker at 800 rpm, 90°C for 20 min.

Gas chromatography-mass spectrometry (GC-MS)

For GC-MS analysis, an Agilent 5860 gas chromatograph was used under the control of MassHunter software (USA). Samples were injected using an Agilent 7893 autosampler. The sample was injected in the splitless mode, with an injected sample volume of 1 µl. Separation was performed on a J&W DB-5MS capillary column (30 m long, 0.25 mm in diameter, stationary phase film thickness 1 µm). The carrier gas was helium; constant flow was 1 mL/min; evaporator temperature was 250°C. The column temperature regime started with an initial temperature of 70°C, then a linear increase at a rate of 6°C/min to 320°C [32]. The chromatogram was registered using an Agilent 5975 mass-selective detector. The mass range was 50–700 m/z. The temperature of the ion source was 230°C and that of the quadrupole mass filter was 150°C. The chromatographic equipment of the Resource Center of St. Petersburg State University "Development of molecular and cellular technologies" was used for this research.

Interpretation of GC-MS results

The resulting chromatograms were processed using the MS-DIAL 4.9 program [33]. We used the Golm Metabolome Database (GMD) library (Germany) [34], as well as the library of the Laboratory of Analytical Phytochemistry of the Botanical Institute of the Russian Academy of Sciences. Additionally, the AMDIS and NIST MSEARCH NIST (National Institute of Standards and Technology, USA) programs, in combination with the NIST08 libraries, were used to identify the mass spectra. The identification of metabolites was performed by the similarity of the mass spectra with the library ones and by the retention indices (RI). The RI was determined by calibration using saturated hydrocarbons. The content of metabolites was normalized to the internal standard tricosane (normal hydrocarbon C_{23}).

Statistical analysis

Analysis of metabolomic data was performed using R4.2.1 "Funny-Looking Kid" [35]. The data were normalized to the sample median. Outliers were detected and excluded based on the Dixon test in the outliers package [36]. Data were taken logarithmically and standardized. If a compound was not found in a particular sample but was present in other replicates, this was considered a technical error, and imputation was performed using the KNN (k-nearest neighbors) method using the impute package [37]. Principal component analysis (PCA) was performed using pcaMethods [38]. OPLS-DA (discriminant analysis by orthogonal projections on latent structures) was performed using the ropls package. Factor loadings of the predictive component and VIP (variable importance in the projection) were used to assess the statistical relationship between the variables and the factor of interest [39]. Models were evaluated by R^2Y predictive component and Q^2Y probability ($p < 0.01$). To avoid bias due to group imbalance, weighted centering was used [40]. Heat maps were plotted using the ComplexHeatmap package [41].

The fgsea algorithm was used for metabolite set enrichment analysis (MSEA) [42]. The sets of metabolites for biochemical pathways were downloaded from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Japan) [43] via the KEGGREST package [44], and *Arabidopsis thaliana* was chosen as a reference organism. Graphs were plotted using the Cytoscape software [45].

RESULTS

Using GC-MS, we determined the metabolomic profiles of the leaves of three fireweed species, namely the mesophyte *Epilobium (Chamaenerion) angustifolium* and the hydrophytes *E. hirsutum* and *E. palustre*. The resulting profiles included approximately 360 compounds, approximately 70 of which were identified in databases, and a class was determined for approximately 50 more compounds (Fig. 1). Sugars (64), including pentoses (8), hexoses (25), and oligosaccharides (20), as well as their derivatives, such as sugar alcohols (3) and sugar acids (8), were most widely represented in the profiles obtained. Sixteen amino acids have been identified, including 13 proteinogenic amino acids. We also identified approximately 20 carboxylic acids, including intermediates of the Krebs cycle (6), metabolites of glycolysis and fermentation (5), as well as a small number of free fatty acids, acylglycerols, sterols, amines, and phenolic compounds. Fig. 1 shows the heat maps combined with VIP bar chart from OPLS-DA models comparing the mesophyte with hydrophytes (top row) and hydrophytic species with each other (bottom row).

We used PCA to identify similarities and differences between metabolomes as presented in Fig. 2. According to the principal component 1 (PC1), which explains 28.6% of the variance, the profiles of *E. angustifolium* metabolites differed significantly from those of the

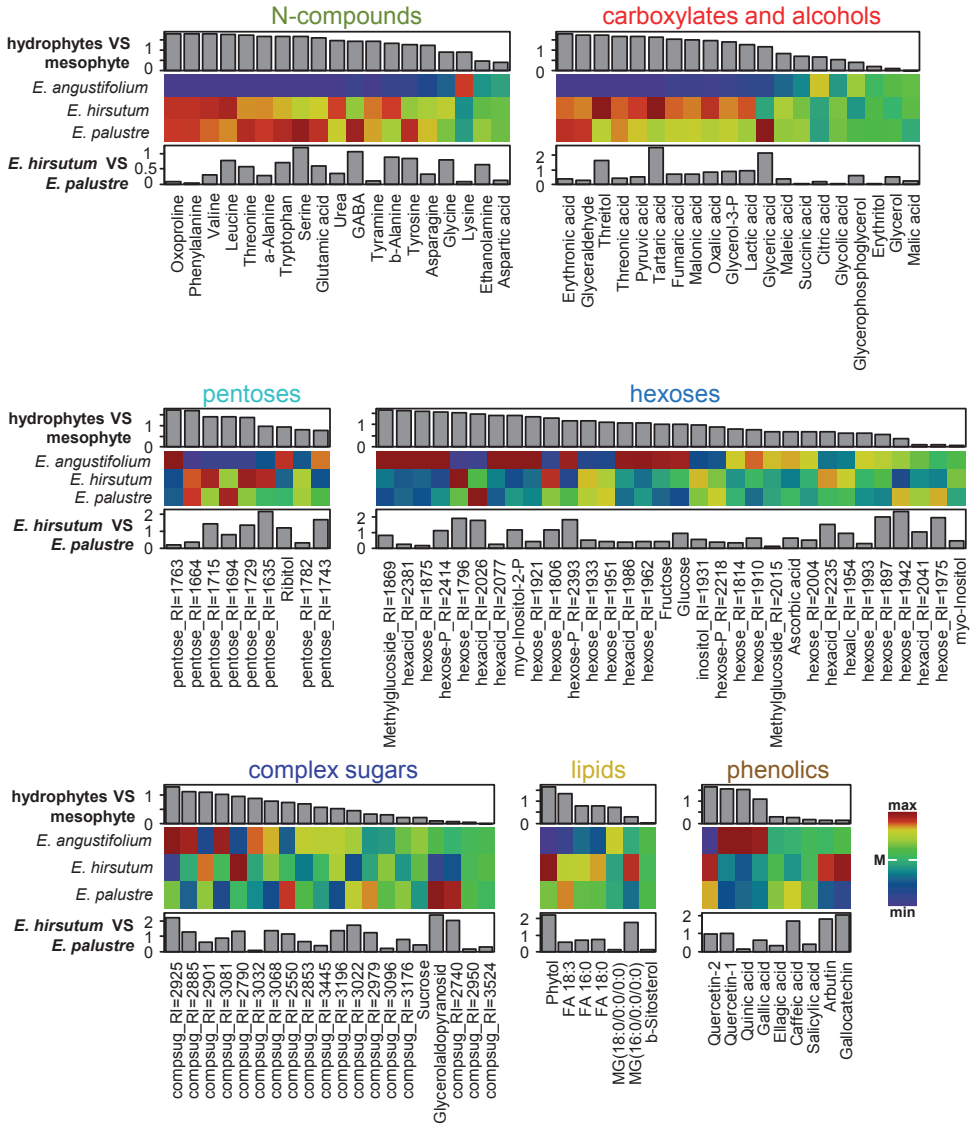

Fig. 1. Heatmap of mean normalized content identified metabolites. Barplots — VIPs from OPLS-DA models for comparison: above — hydrophytes and mesophyte, under — *E. hirsutum* and *E. palustre*. In metabolite names: RI — retention index, -P — phosphate, compsug- complex sugars or molecules with sugar parts, FA — fatty acid, MG — monoacylglycerol

Рис. 1. Тепловая карта среднего нормализованного содержания идентифицированных метаболитов. Столбчатые диаграммы — VIP из моделей OPLS-DA для сравнения: вверху — гидрофитов и мезофита, внизу — *E. hirsutum* и *E. palustre*. В названиях метаболитов: compsug — сложные сахара или молекулы с сахарными частями, RI — индекс удерживания, -P — фосфат, FA — жирная кислота, MG — моноацилглицерин

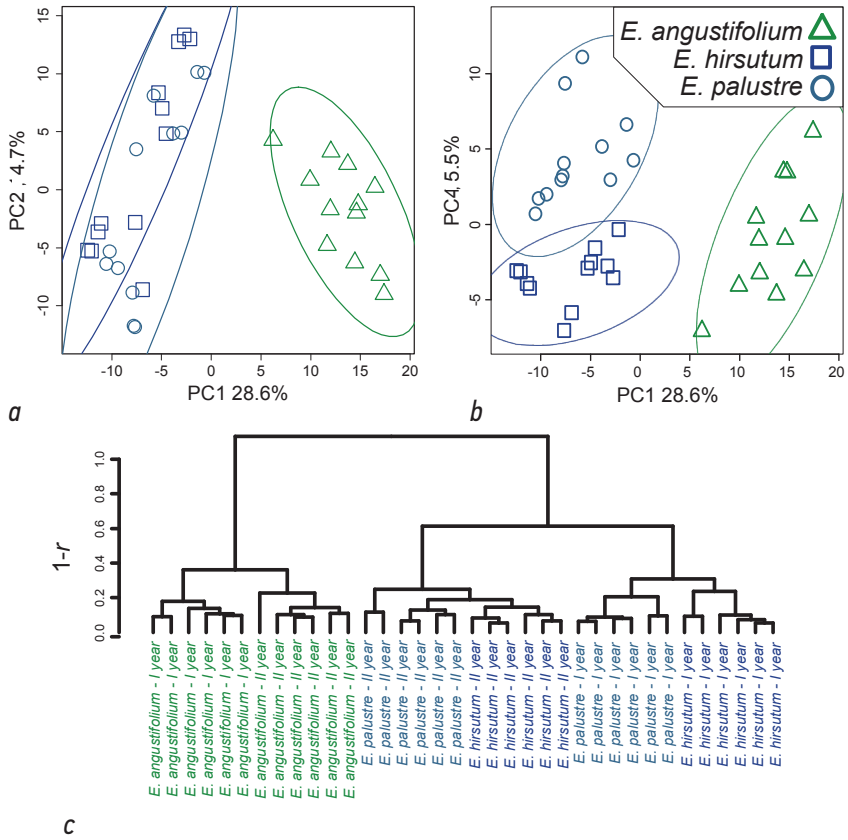

Fig. 2. Unsupervised analysis of metabolite profiles from three *Epilobium* species sampled at two years. *a*, *b* — PCA score plots, ellipses — 95% CI; *c* — dendrogram of hierarchical clustering [with Pearson distance (1-*r*), Ward method]

Рис. 2. Анализ без обучения профилей метаболитов трех видов *Epilobium*, собраных в течение двух лет: *a*, *b* — графики счетов методом главных компонет (ГК), эллипсы — 95 % доверительные интервалы; *c* — дендрограмма иерархической кластеризации (с расстоянием Пирсона (1-*r*), агломерация методом Уорда)

other two species, which were practically indistinguishable from each other (Fig. 2*a*). However, *E. palustre* and *E. hirsutum* differed in PC4 (5.5% variance, Fig. 2*b*). We next performed a hierarchical cluster analysis using the Spearman distance, 1-rho, where rho is the Spearman correlation coefficient. This approach reduced the influence of the normalization method on the result (Fig. 2*c*). However, even in this case, *E. angustifolium* was clustered separately from the other species. Accessions of *E. angustifolium* were also grouped according to the year of collection (1 or 2). The profiles of *E. palustre* and *E. hirsutum* were clustered, first, according to the year of sampling and, secondly, according to the species (Fig. 2*c*). From these data, it can be concluded that, at the level of the metabolome, the mesophytic *E. angustifolium* clearly differed from the other two hydrophyte species. Differences in the metabolomes of *E. hirsutum* and *E. palustre* were also present, but they were less apparent and were not observed with annual changes.

In order to identify which metabolites accumulated at different levels between mesophytes and hydrophytes, we used discriminant analysis by the method of orthogonal projections onto latent structures (OPLS-DA). The model constructed by OPLS-DA included two orthogonal components. Approximately 28% of the variance was associated with the predictive component, reflecting the differences between the groups ($Q^2Y = 0.78$). Metabolites with a VIP value of 1 or greater can be considered to be reliably associated with differences between hydrophytes and mesophytes. The results are presented in Fig. 1 (top row of bar chart) of the VIP bar charts for the above model. A characteristic feature of the hydrophytes was a greater accumulation of a wide range of nitrogen-containing compounds, primarily the amino acids 5-oxoproline, phenylalanine, valine, leucine, threonine, α- and β-alanine, tryptophan, serine, glutamate, asparagine, and GABA. Moreover, *E. hirsutum* and *E. palustre* contained larger amounts of organic acids, including intermediates of the Krebs cycle (fumarate and succinate), glycolysis (glycerate, pyruvate), and fermentation (lactate). On the other hand, the mesophyte was characterized by higher levels of sugars, including glucose, fructose, hexose phosphates, and ascorbate. Lipophilic compounds generally did not differ between ecological groups, except for phytol and linolenic acid which accumulated in greater amounts in *E. angustifolium*. Among secondary metabolites, *E. angustifolium* accumulated more quinic and gallic acids.

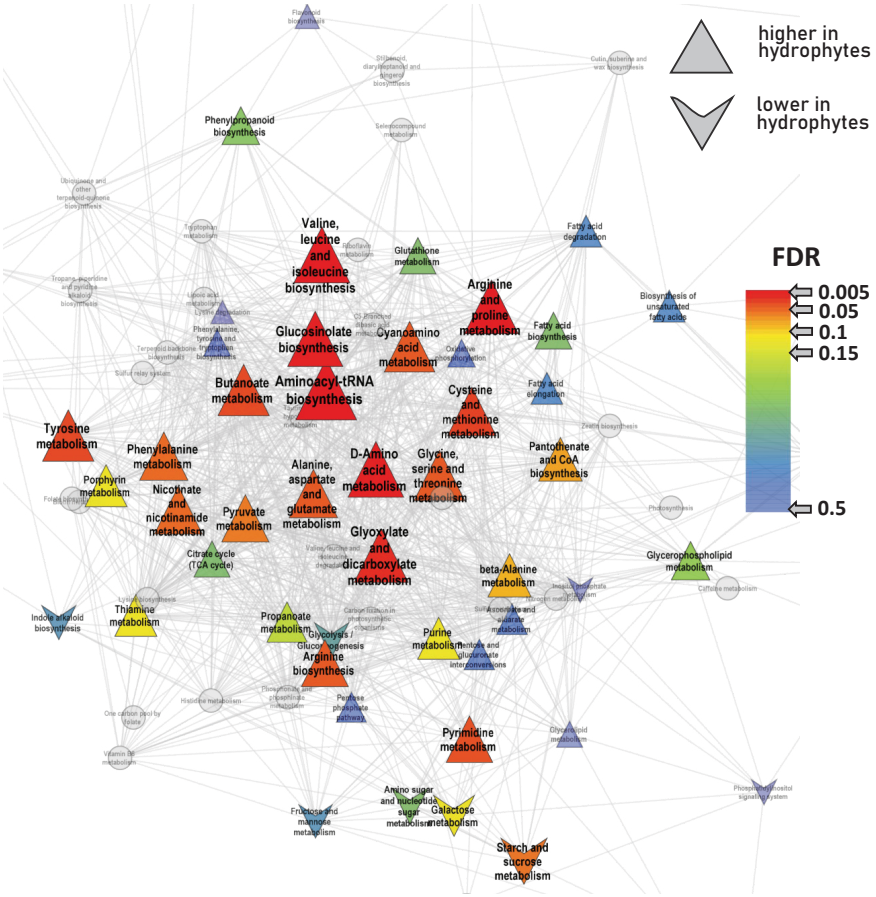

Fig. 3. Metabolite sets enrichment analysis based on loadings from OPLS-DA classification of hydrophytes and mesophyte. Nodes are the paths extracted from KEGG. If the paths share metabolites, then they are connected by edge. Nodes attract with each other in dependence of number of common metabolites. Color — significance of influence on this pathway, size — strength of influence (|NES|). NES — normalized enrichment score. FDR — false discovery rate

Рис. 3. Анализ обогащения наборов метаболитов на основе факторных загрузок из OPLS-DA-моделей классификации гидрофитов и мезофита. Узлы — пути, полученные из базы KEGG. Если пути имеют общие метаболиты, то они соединены ребром. Края притягиваются в зависимости от количества общих метаболитов. Цвет — значимость влияния на этот путь, размер — сила влияния (|NES|). NES — нормализованная оценка обогащения (normalized enrichment score), FDR — уровень ложноположительных результатов (false discovery rate)

Figure 1 (bottom row of bar graphs) presents a comparison of the metabolomes of the two hydrophytes *E. hirsutum* and *E. palustre.* The model constructed using OPLS-DA included one orthogonal component. Approximately 12% of the variance was associated with the predictive component, reflecting the differences between the groups (Q^2Y = 0.75). Unlike ecological differences, interspecies differences in hydrophytes were not exclusive to any of the compound groups. *E. hirsutum* exhibited higher content of leucine, β-alanine, urea, organic acids (with the exception of glycerate), pentoses, lipophilic compounds, arbutin, and gallocatechin. On the other hand, *E. palustre* accumulated more amino acids, especially serine, tyrosine, and GABA, as well as glyceric acid and caffeic acid.

To identify the biochemical pathways most significantly associated with environmental differences, we performed an enrichment analysis (MSEA) using for metabolite ranking the loadings of the predictive component from the OPLS-DA model and metabolite sets for pathways obtained from the KEGG database. The results of this analysis are presented in Fig. 3. The hydrophytes accumulated more metabolites in their leaves, particularly those involved in the metabolism of organic acids, amino acids, and other nitrogen-containing compounds. On the other hand, hydrophyte sugar metabolism was lowered compared with *E. angustifolium*.

To compare differences between hydrophytes and mesophytes with species differences between two hydrophytes, we constructed a SUS-plot (shared and unique structures), where metabolites are presented in the space of factor loadings of the two OPLS models described above (Fig. 4). A very weak relationship was registered (rho = 0.11, p = 0.04). Because the plants were collected over two years, we also constructed 3 OPLS-DA models to compare yearly samples for each species. The proportion of variance (%) and Q^2Y of the predictive component were 38% and 0.91 for *E. hirsutum*, 33% and 0.89 for

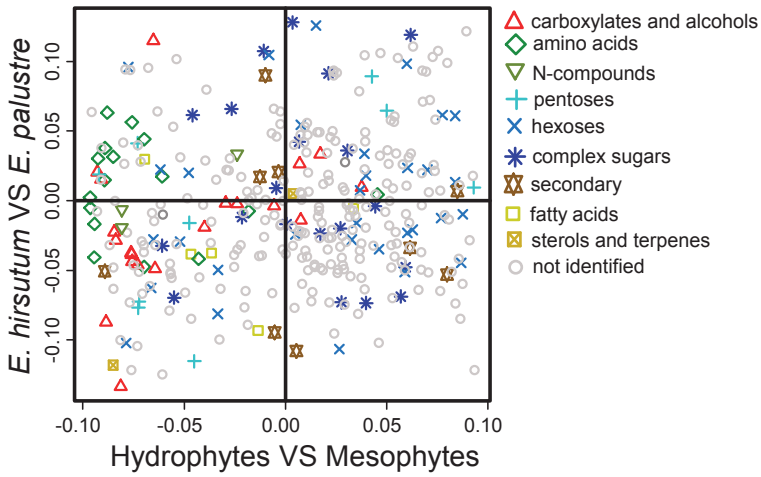

Fig. 4. Comparison of differences between hydrophytes and mesophyte and between two hydrophytes. SUS (shared and unique structures) plot in the space of the loadings from two OPLS-DA models. Positive loadings correspond to a higher content in mesophyte and in *E. palustre*

Рис. 4. Сравнение различий между гидрофитами и мезофитом и между двумя гидрофитами. График общих и уникальных структур (SUS, shared and unique structures) в пространстве нагрузок от двух OPLS-DA-моделей. Положительные значения нагрузок соответствуют большему уровню содержания в мезофите и у *Epilobium palustre*

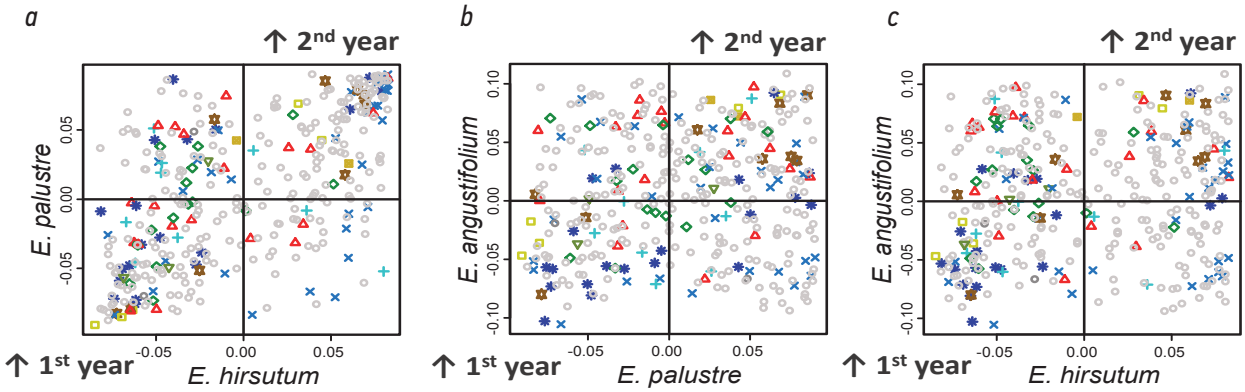

Fig. 5. Comparison of annual changes in metabolite profiles of three *Epilobium* species: *a* — comparison *E. palustre* vs *E. hirsutum*; *b* — comparison *E. angustifolium* vs *E. palustre*; *c* — comparison *E. angustifolium* vs *E. hirsutum*. SUS plots in the loadings space of the corresponding OPLS-DA models. Positive loadings correspond to a higher level in the second year of observations

Рис. 5. Сравнение годичных изменений профилей метаболитов трех видов кипреев: *a* — сравнение *E. palustre* и *E. hirsutum*; *b* — сравнение *E. angustifolium* и *E. palustre*; *c* — сравнение *E. angustifolium* и *E. hirsutum*. Графики общих и уникальных структур (SUS) в пространстве нагрузок соответствующих OPLS-DA моделей. Положительные значения нагрузок соответствуют большему уровню содержания на второй год наблюдений

E. palustre, and 23% and 0.86 for *E. angustifolium*, respectively. Thus, annual changes in the hydrophytic fireweeds were more pronounced than in the mesophytic fireweed. As seen in Fig. 5*a*, annual changes in the two hydrophytes *E. hirsutum* and *E. palustre* were very similar (rho = 0.7, $p < 10^{-15}$). On the other hand, the annual changes in *E. angustifolium* had little in common with those of the hydrophytes (Fig. 5*b, c*). A common feature we observed in fireweeds is a greater accumulation of complex sugars against a decrease in secondary compounds in year 1 of collection. In the case of hydrophytes, there was a trend toward a greater accumulation of amino acids in the first year.

DISCUSSION

Metabolomics is a modern and highly informative method for analyzing adaptation processes in living systems under the influence of various environmental factors. Over the past 20 years, there has been an increase in the studies of plant metabolomes under the influence of drought, salinity, and unfavorable temperatures. However, fewer than 5% of these works related to the study of the effect of oxygen deficiency [46]. The use of various analytical platforms [GC-MS, liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR)] enabled the identification of changes in sugar metabolism and Krebs cycle and the detection

of alanine, succinate, GABA, and polyamine accumulation [18, 22, 25, 26, 47–49]. Changes in the metabolome under oxygen deficiency were also characterized in the hydrophytes *Zostera marina* [23] and *Potamogeton anguillanus* [24].

Metabolomic profiling of two fireweed species, *E. angustifolium* [50] and *E. hirsutum* [51], was previously performed by LC-MS. However, the study only searched for biologically active secondary compounds for pharmacological use and did not aim to compare the species with each other. In the present study, we detected the same organic acids and secondary metabolites identified in the previous study. Using GC-MS, we revealed significant differences in the metabolic profile of the leaves of mesophytic *E. angustifolium* from the profiles of hydrophytic *E. hirsutum* and *E. palustre* (Fig. 1, 2). As in the case of other hypoxia-tolerant plants, hydrophytic fireweeds exhibited stimulation of nitrogen metabolism (Fig. 3) and the accumulation of a number of amino acids, including 5-oxoproline, α-alanine, glutamate, asparagine, and GABA. Proline is a stress metabolite involved in osmotic adjustment under drought and salinity conditions (i. e. water retention in the cell and protection of macromolecules from denaturation) [52]. In our study, the level of this amino acid did not differ significantly in the studied species. Nevertheless, another important function of proline during stress is antioxidant protection. In this case, it can be oxidized to 5-oxoproline [52], which we found to be significantly elevated in the leaves of hydrophytes (Fig. 1). Oxidative damage is an important mechanism of stress during oxygen deficiency. This effect is most pronounced during re-aeration after hypoxia and anoxia but can also develop during root hypoxia [9, 11, 53, 54]. Wetland plants are characterized by a more active antioxidant system [11, 55]. Thus, the presence of oxoproline may indicate oxidative stress in the tissues of hydrophytic fireweeds which is counteracted by proline. Elevated levels of alanine, glutamate, and GABA indicate increased anaplerotic pathways of NAD(P)H reoxidation in *E. hirsutum* and *E. palustre*, particularly the GABA shunt [5, 6, 9, 21]. This metabolic pathway is a bypass branch of the Krebs cycle where 2-oxoglutarate is aminated to glutamate, glutamate is decarboxylated to GABA, and GABA is converted to succinic semialdehyde [56]. This reaction is coupled with the transamination of pyruvic acid to alanine. Succinic semialdehyde is then converted to succinic acid, which is then converted to fumarate. We detected both dicarboxylic acids of the Krebs cycle (succinate and fumarate) in the leaves of *E. hirsutum* and *E. palustre* (Fig. 1) and MSEA showed stimulation of dicarboxylic acid metabolism (Fig. 3). In addition, an increase in proteinogenic amino acids abundance may indicate a stimulation of nitrogen metabolism, including the reduction of nitrate, which can be used as an alternative terminal electron acceptor instead of atmospheric oxygen (nitrate respiration). Elevated amino acid levels may also reflect increased synthesis of amino acids and anaerobic stress proteins during oxygen lack [6, 9]. The levels of glyceric, pyruvic, and lactic acids were also increased in the tissues of hydrophytes. Accumulation of these compounds may be a marker of glycolytic processes in a hypoxic environment that favor lactic acid or alcoholic fermentation. Unfortunately, the method of analysis we used did not enable the detection of the level of other important anaerobic metabolites (acetaldehyde and ethanol) that evaporate during sample preparation and derivatization. The features of leaf metabolomes of hydrophytic fireweed species we found were similar to those of other flood-tolerant plants, such as rice [20, 25], *Zostera marina* [23], and *Potamogeton anguillanus* [24].

The mesophytic *E. angustifolium* metabolome was characterized by the accumulation of sugars, probably due to the stimulation of carbohydrate metabolism (Fig. 1, 3).

Comparison of the metabolomes of two hydrophytic species with each other showed that the differences were not specific on any class of compounds and none of the biochemical pathways were significantly associated with the differences in the species (Fig. 1, 5). Differences in the secondary compounds and complex sugars (including glycosides of secondary compounds) in *E. hirsutum* and *E. palustre* is noteworthy. These findings suggest that the biochemical divergence of ecologically similar species occurs primarily via secondary metabolism.

A comparison of differences between hydrophytes and mesophytes with the species differences between the two hydrophytes did not reveal a direct relationship between them (Fig. 4). Thus, biochemical divergence within one ecotope may not be similar to changes due to adaptation.

Comparison of annual differences in metabolomic profiles for each species revealed commonalities in the changes in *E. hirsutum* and *E. palustre* (Fig. 5*a*). Moreover, the annual differences were stronger than the interspecific ones, as the profiles were clustered according to the year of collection more than according to the species (Fig. 2*c*). Thus, growth in the same ecotope and same environmental conditions, together with phylogenetic proximity, triggers similar metabolic responses. In mesophytic *E. angustifolium*, annual changes differed from the hydrophytes (Fig. 5*b, c*).

In summary, significant differences in the leaf metabolic profiles of mesophytic *E. angustifolium* and hydrophytic *E. hirsutum* and *E. palustre* were revealed. The metabolomes of hydrophytic fireweeds were almost identical and were characterized by the accumulation of amino acids, including GABA shunt intermediates, dicarboxylic acids of the Krebs cycle, and metabolites of glycolysis and lactic acid fermentation. These reflect the stimulation of anaerobic respiration, nitrogen

metabolism, and alternative pathways of NAD(P)H reoxidation in these aquatic plants. These changes are also reported to be characteristic of other plants tolerant to oxygen deficiency, suggesting that this set of metabolites can be used to assess the degree of tolerance to hypoxia and anoxia.

It should be taken into consideration that the genus *Epilobium* is quite large and consists of several subsets. *E. hirsutum* and *E. palustre* belong to the *Epilobium* section, while *E. angustifolium* belongs to the *Chamaenerion* section, which some taxonomists, including Russian botanists, distinguish as a separate genus *Chamaenerion* Seg. [29]. Thus, the observed differences in metabolomic profiles may be due to taxonomic differences and not just ecological differences. For future studies, it would be necessary to expand the comparison of metabolomes of closely related hydrophytic and mesophytic plants to a larger number of taxonomic groups.

ADDITIONAL INFORMATION

Author contribution. Thereby, all authors confirm that their authorship complies with the international ICMJE criteria (all authors have made a significant contribution to the development of the concept, research, and preparation of the article, as well as read and approved the final version before its publication). Personal contribution of the authors: R.K. Puzanskiy — GC-MS, data analysis, writing the main part of the text; P.D. Smirnov — experimental design, collecting and preparation of samples, writing the main part of the text; S.A. Vanisov and M.D. Dubrovskiy — collecting and preparation of samples; A.L. Shavarda — GC-MS, data analysis; M.F. Shishova — data analysis, literature review, making final edits; V.V. Yemelyanov — experimental design, collecting and preparation of samples, GC–MS, data analysis, writing the main part of the text, literature review, making final edits, funding acquisition.

Competing interests. The authors declare no conflict of interests.

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REFERENCES

1. Dennis ES, Dolferus R, Ellis M, et al. Molecular strategies for improving waterlogging tolerance in plants. *J Exp Bot*. 2000;51(342):89–97. DOI: 10.1093/jexbot/51.342.89

2. Fukao T, Barrera-Figueroa BE, Juntawong P, Peña-Castro JM. Submergence and waterlogging stress in plants: A review highlighting research opportunities and understudied aspects. *Front Plant Sci*. 2019;10:340. DOI: 10.3389/fpls.2019.00340

3. Voesenek LACJ, Colmer TD, Pierik R, et al. How plants cope with complete submergence. *New Phytol*. 2006;170(2):213–226. DOI: 10.1111/j.1469-8137.2006.01692.x

4. Bailey-Serres J, Voesenek LACJ. Flooding stress: Acclimations and genetic diversity. *Annu Rev Plant Biol*. 2008;59:313–339. DOI: 10.1146/annurev.arplant.59.032607.092752

5. Crawford RMM. Studies in plant survival. Anderson DJ, Greic-Smith P, Pitelka FA, editors. *Ecological case histories of plant adaptation to adversity. Studies in ecology. Vol. 11*. Oxford; London; Edinburgh; Boston; Palo Alto; Melbourne: Blackwell Scientific Publications, 1989. 296 p.

6. Chirkova TV. Rastenie i anaehrobioz. *Vestnik of Saint Petersburg University: Series 3: Biology*. 1998;(2):41–52. (In Russ.)

7. Vartapetian BB, Jackson MB. Plant adaptations to anaerobic stress. *Ann Bot*. 1997;79(S1):3–20. DOI: 10.1093/oxfordjournals.aob.a010303

8. Chirkova TV, Walter G, Leffer S, Novitskaya LO. Chloroplasts and mitochondria in the leaves of wheat and rice seedlings exposed to anoxia and long-term darkness: Some characteristics of organelle state. *Russ J Plant Physiol*. 1995;42(3):321–329.

9. Chirkova T, Yemelyanov V. The study of plant adaptation to oxygen deficiency in Saint Petersburg University. *Biol Commun*. 2018;63(1):17–31. DOI: 10.21638/spbu03.2018.104

10. Blokhina OB, Chirkova TV, Fagerstedt KV. Anoxic stress leads to hydrogen peroxide formation in plant cells. *J Exp Bot*. 2001;52(359):1179–1190. DOI: 10.1093/jexbot/52.359.1179

11. Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: A review. *Ann Bot*. 2003;91(2):179–194. DOI: 10.1093/aob/mcf118

12. Blokhina O, Fagerstedt KV. Reactive oxygen species and nitric oxide in plant mitochondria: Origin and redundant regulatory systems. *Physiol Plant*. 2010;138(4):447–462. DOI: 10.1111/j.1399-3054.2009.01340.x

13. Chirkova TV, Novitskaya LO, Blokhina OB. Lipid peroxidation and antioxidant systems under anoxia in plants differing in their tolerance to oxygen deficiency. *Russ J Plant Physiol*. 1998;45(1):55–62.

14. Blokhina OB, Fagerstedt KV, Chirkova TV. Relationships between lipid peroxidation and anoxia tolerance in a range of species during post-anoxic reaeration. *Physiol Plant*. 1999;105(4):625–632. DOI: 10.1034/j.1399-3054.1999.105405.x

15. Shikov AE, Lastochkin VV, Chirkova TV, et al. Post-anoxic oxidative injury is more severe than oxidative stress induced by chemical agents in wheat and rice plants. *Acta Physiol Plant*. 2022;44(9):90. DOI: 10.1007/s11738-022-03429-z

16. Sweetlove LJ, Dunford R, Ratcliffe RG, Kruger NJ. Lactate metabolism in potato tubers deficient in lactate dehydrogenase activity. *Plant Cell Environ*. 2000;23(8):873–881. DOI: 10.1046/j.1365-3040.2000.00605.x

17. Licausi F, Perata P. Low oxygen signaling and tolerance in plants. *Adv Bot Res*. 2009;50:139–198. DOI: 10.1016/S0065-2296(08)00804-5

18. van Dongen JT, Frohlich A, Ramirez-Aguilar SJ, et al. Transcript and metabolite profiling of the adaptive response to mild decreases in oxygen concentration in the roots of arabidopsis plants. *Ann Bot*. 2009;103(2):269–280. DOI: 10.1093/aob/mcn126

19. Rocha M, Licausi F, Araujo WL, et al. Glycolysis and the tricarboxylic acid cycle are linked by alanine aminotransferase during hypoxia induced by waterlogging of *Lotus japonicas*. *Plant Physiol*. 2010;152(3):1501–1513. DOI: 10.1104/pp.109.150045

20. Barding GA Jr, Fukao T, Beni S, et al. Differential metabolic regulation governed by the rice *SUB1A* gene during submergence stress and identification of alanylglycine by ^{1}H NMR spectroscopy. *J Proteome Res*. 2012;11(1):320–330. DOI: 10.1021/pr200919b
21. Antonio C, Päpke C, Rocha M, et al. Regulation of primary metabolism in response to low oxygen availability as revealed by carbon and nitrogen isotope redistribution. *Plant Physiol*. 2016;170(1):43–56. DOI: 10.1104/pp.15.00266
22. Herzog M, Fukao T, Winkel A, et al. Physiology, gene expression, and metabolome of two wheat cultivars with contrasting submergence tolerance. *Plant Cell Environ*. 2018;41(7):1632–1644. DOI: 10.1111/pce.13211
23. Hasler-Sheetal H, Fragner L, Holmer M, Weckwerth W. Diurnal effects of anoxia on the metabolome of the seagrass *Zostera marina*. *Metabolomics*. 2015;11(5):1208–1218. DOI: 10.1007/s11306-015-0776-9
24. Parveen M, Miyagi A, Kawai-Yamada M, et al. Metabolic and biochemical responses of *Potamogeton anguillanus* Koidz. (Potamogetonaceae) to low oxygen conditions. *J Plant Physiol*. 2019;232:171–179. DOI: 10.1016/j.jplph.2018.11.023
25. Locke AM, Barding GA Jr, Sathnur S, et al. Rice *SUB1A* constrains remodelling of the transcriptome and metabolome during submergence to facilitate post-submergence recovery. *Plant Cell Environ*. 2018;41(4):721–736. DOI: 10.1111/pce.13094
26. Coutinho ID, Henning LMM, Döpp SA, et al. Identification of primary and secondary metabolites and transcriptome profile of soybean tissues during different stages of hypoxia. *Data in Brief*. 2018;21:1089–1100. DOI: 10.1016/j.dib.2018.09.122
27. theplantlist.org [Internet]. *The Plant List* [cited 2022 Nov 20]. Available at: http://theplantlist.org/1.1/browse/A/Onagraceae/Epilobium/
28. mobot.org [Internet]. *Angiosperm phylogeny website, version 14* [cited 2022 Nov 20]. Available at: http://www.mobot.org/MOBOT/Research/APweb/orders/myrtalesweb2.htm#Onagraceae
29. Maevskii PF. *Flora srednei polosy evropeiskoi chasti Rossii. 11th edition*. Moscow: Tovarishchestvo nauchnykh izdanii KMK, 2014. 635 p. (In Russ.)
30. Ronzhina DA. Ecological differentiation between invasive and native species of the genus *Epilobium* in riparian ecosystems is associated with plant functional traits. *Russ J Biol Invas*. 2020;(1):38–51. (In Russ.)
31. Chirkov YI. *Osnovy agrometeorologii*. Leningrad: Gidrometeoizdat, 1988. 248 p. (In Russ.)
32. Puzanskiy RK, Yemelyanov VV, Shavarda AL, et al. Age- and organ-specific differences of potato (*Solanum phureja*) plants metabolome. *Russ J Plant Physiol*. 2018;65(6):813–823. DOI: 10.1134/S1021443718060122
33. Lai Z, Tsugawa H, Wohlgemuth G, et al. Identifying metabolites by integrating metabolome databases with mass spectrometry cheminformatics. *Nat Methods*. 2018;15:53–56. DOI: 10.1038/nmeth.4512
34. Hummel J, Selbig J, Walther D, Kopka J. The Golm Metabolome Database: a Database for GC-MS based metabolite profiling. Nielsen J, Jewett MC, editors. *Metabolomics. Vol. 18: Topics in Current Genetics*. Berlin; Heidelberg: Springer. 2007. P. 75–95. DOI: 10.1007/4735_2007_0229
35. r-project.org [Internet]. *R Core Team. R: The R Project for Statistical Computing* [cited 2022 Nov 20]. Available at: https://www.r-project.org/
36. CRAN.R-project.org [Internet]. *Komsta L. outliers: Tests for Outliers. R package version 0.15, 2022* [cited 2022 Nov 20]. Available at: https://CRAN.R-project.org/package=outliers
37. bioconductor.org [Internet]. *Hastie T, Tibshirani R, Narasimhan B, Chu G. Impute: Imputation for microarray data. R package version 1.70.0. 2022*. Available at: https://bioconductor.org/packages/release/bioc/html/impute.html
38. Stacklies W, Redestig H, Scholz M, et al. pcaMethods — a Bioconductor package providing PCA methods for incomplete data. *Bioinformatics*. 2007;23(9):1164–1167. DOI: 10.1093/bioinformatics/btm069
39. Thevenot EA, Roux A, Xu Y, et al. Analysis of the human adult urinary metabolome variations with age, body mass index and gender by implementing a comprehensive workflow for univariate and OPLS statistical analyses. *J Proteome Res*. 2015;14(8):3322–3335. DOI: 10.1021/acs.jproteome.5b00354
40. Brereton RG, Lloyd GR. Partial least squares discriminant analysis: taking the magic away. *J Chemom*. 2013;28(4):213–225. DOI: 10.1002/cem.2609
41. Gu Z, Eils R, Schlesner M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics*. 2016;32(18):2847–2849. DOI: 10.1093/bioinformatics/btw313
42. Korotkevich G, Sukhov V, Sergushichev A. Fast gene set enrichment analysis. *bioRxiv*. 2019;1–40. DOI: 10.1101/060012
43. Kanehisa M, Furumichi M, Sato Y, et al. KEGG for taxonomy-based analysis of pathways and genomes. *Nucleic Acids Res*. 2022;gkac963. DOI: 10.1093/nar/gkac963
44. bioconductor.org [Internet]. *Tenenbaum D, Maintainer B. KEGGREST: Client-side REST access to the Kyoto Encyclopedia of Genes and Genomes (KEGG). 2022. R package version 1.36.2*. Available at: https://www.bioconductor.org/packages/release/bioc/html/KEGGREST.html
45. Shannon P, Markiel A, Ozier O, et al. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003;13(11):2498–2504. DOI: 10.1101/gr.1239303
46. Xu Y, Fu X. Reprogramming of plant central metabolism in response to abiotic stresses: A metabolomics view. *Int J Mol Sci*. 2022;23(10):5716. DOI: 10.3390/ijms23105716
47. Shingaki-Wells RN, Huang S, Taylor NL, et al. Differential molecular responses of rice and wheat coleoptiles to anoxia reveal novel metabolic adaptations in amino acid metabolism for tissue tolerance. *Plant Physiol*. 2011;156(4):1706–1724. DOI: 10.1104/pp.111.175570
48. Mustroph A, Barding GA Jr, Kaiser KA, et al. Characterization of distinct root and shoot responses to low-oxygen stress in Arabidopsis with a focus on primary C- and N-metabolism. *Plant Cell Environ*. 2014;37(10):2366–2380. DOI: 10.1111/pce.12282
49. Fukushima A, Kuroha T, Nagai K, et al. Metabolite and phytohormone profiling illustrates metabolic reprogramming as an escape strategy of deepwater rice during partially submerged stress. *Metabolites*. 2020;10(2):68. DOI: 10.3390/metabo10020068
50. Dacrema M, Sommella E, Santarcangelo C, et al. Metabolic profiling, *in vitro* bioaccessibility and *in vivo* bioavailability of a commercial bioactive *Epilobium angustifolium* L. extract. *Biomed Pharmacother*. 2020;131:110670. DOI: 10.1016/j.biopha.2020.110670
51. Ak G, Zengin G, Mahomoodally MF, et al. Shedding light into the connection between chemical components and biological effects of extracts from *Epilobium hirsutum*: Is it a potent source of bioactive agents from natural treasure? *Antioxidants*. 2021;10(9):1389. DOI: 10.3390/antiox10091389
52. Matysik J, Alia A, Bhalu B, Mohanty P. Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. *Curr Sci*. 2002;82(5):525–532.

53. Tamang BG, Fukao T. Plant adaptation to multiple stresses during submergence and following desubmergence. *Int J Mol Sci*. 2015;16(12):30164–30180. DOI: 10.3390/ijms161226226

54. Shikov AE, Chirkova TV, Yemelyanov VV. Post-anoxia in plants: reasons, consequences, and possible mechanisms. *Russ J Plant Physiol*. 2020;67(1):45–59. DOI: 10.1134/S1021443720010203

55. Yemelyanov VV, Lastochkin VV, Prikazyuk EG, Chirkova TV. Activities of catalase and peroxidase in wheat and rice plants under conditions of anoxia and post-anoxic aeration. *Russ J Plant Physiol*. 2022;69(6):117. DOI: 10.1134/S1021443722060036

56. Shelp BJ, Bown AW, McLean MD. Metabolism and functions of gamma-aminobutyric acid. *Trends Plant Sci*. 1999;4(11): 446–452. DOI: 10.1016/S1360-1385(99)01486-7

СПИСОК ЛИТЕРАТУРЫ

1. Dennis E.S., Dolferus R., Ellis M., et al. Molecular strategies for improving waterlogging tolerance in plants // J Exp Bot. 2000. Vol. 51, No. 342. P. 89–97. DOI: 10.1093/jexbot/51.342.89

2. Fukao T., Barrera-Figueroa B.E., Juntawong P., Peña-Castro J.M. Submergence and waterlogging stress in plants: A review highlighting research opportunities and understudied aspects // Front Plant Sci. 2019. Vol. 10. ID 340. DOI: 10.3389/fpls.2019.00340

3. Voesenek L.A.C.J., Colmer T.D., Pierik R., et al. How plants cope with complete submergence // New Phytol. 2006. Vol. 170, No. 2. P. 213–226. DOI: 10.1111/j.1469-8137.2006.01692.x

4. Bailey-Serres J., Voesenek L.A.C.J. Flooding stress: Acclimations and genetic diversity // Annu Rev Plant Biol. 2008. Vol. 59. P. 313–339. DOI: 10.1146/annurev.arplant.59.032607.092752

5. Crawford R.M.M. Studies in plant survival. Ecological case histories of plant adaptation to adversity. Studies in ecology / D.J. Anderson, P. Greic-Smith, F.A. Pitelka, editors. Vol. 11. Oxford; London; Edinburgh; Boston; Palo Alto; Melbourne: Blackwell Scientific Publications, 1989. 296 p.

6. Чиркова Т.В. Растение и анаэробиоз // Вестник Санкт-Петербургского университета. Серия 3: Биология. 1998. № 2. С. 41–52.

7. Vartapetian B.B., Jackson M.B. Plant adaptations to anaerobic stress // Ann Bot. 1997. Vol. 79, No. S1. P. 3–20. DOI: 10.1093/oxfordjournals.aob.a010303

8. Chirkova TV, Walter G, Leffer S, Novitskaya LO. Chloroplasts and mitochondria in the leaves of wheat and rice seedlings exposed to anoxia and long-term darkness: Some characteristics of organelle state // Russ J Plant Physiol. 1995. Vol. 42, No. 3. P. 321–329.

9. Chirkova T., Yemelyanov V. The study of plant adaptation to oxygen deficiency in Saint Petersburg University // Biol Commun. 2018. Vol. 63, No. 1. P. 17–31. DOI: 10.21638/spbu03.2018.104

10. Blokhina O.B., Chirkova T.V., Fagerstedt K.V. Anoxic stress leads to hydrogen peroxide formation in plant cells // J Exp Bot. 2001. Vol. 52, No. 359. P. 1179–1190. DOI: 10.1093/jexbot/52.359.1179

11. Blokhina O., Virolainen E., Fagerstedt K.V. Antioxidants, oxidative damage and oxygen deprivation stress: A review // Ann Bot. 2003. Vol. 91, No. 2. P. 179–194. DOI: 10.1093/aob/mcf118

12. Blokhina O., Fagerstedt K.V. Reactive oxygen species and nitric oxide in plant mitochondria: Origin and redundant regulatory systems // Physiol Plant. 2010. Vol. 138, No. 4. P. 447–462. DOI: 10.1111/j.1399-3054.2009.01340.x

13. Chirkova T.V., Novitskaya L.O., Blokhina O.B. Lipid peroxidation and antioxidant systems under anoxia in plants differing in their tolerance to oxygen deficiency // Russ J Plant Physiol. 1998. Vol. 45, No. 1. P. 55–62.

14. Blokhina O.B., Fagerstedt K.V., Chirkova T.V. Relationships between lipid peroxidation and anoxia tolerance in a range of species during post-anoxic reaeration // Physiol Plant. 1999. Vol. 105, No. 4. P. 625–632. DOI: 10.1034/j.1399-3054.1999.105405.x

15. Shikov A.E., Lastochkin V.V., Chirkova T.V., et al. Post-anoxic oxidative injury is more severe than oxidative stress induced by chemical agents in wheat and rice plants // Acta Physiol Plant. 2022. Vol. 44, No. 9. ID90. DOI: 10.1007/s11738-022-03429-z

16. Sweetlove L.J., Dunford R., Ratcliffe R.G., Kruger N.J. Lactate metabolism in potato tubers deficient in lactate dehydrogenase activity // Plant Cell Environ. 2000. Vol. 23, No. 8. P. 873–881. DOI: 10.1046/j.1365-3040.2000.00605.x

17. Licausi F., Perata P. Low oxygen signaling and tolerance in plants // Adv Bot Res. 2009. Vol. 50. P. 139–198. DOI: 10.1016/S0065-2296(08)00804-5

18. van Dongen J.T., Frohlich A., Ramirez-Aguilar S.J., et al. Transcript and metabolite profiling of the adaptive response to mild decreases in oxygen concentration in the roots of arabidopsis plants // Ann Bot. 2009. Vol. 103, No. 2. P. 269–280. DOI: 10.1093/aob/mcn126

19. Rocha M., Licausi F., Araujo W.L., et al. Glycolysis and the tricarboxylic acid cycle are linked by alanine aminotransferase during hypoxia induced by waterlogging of *Lotus japonicas* // Plant Physiol. 2010. Vol. 152, No. 3. P. 1501–1513. DOI: 10.1104/pp.109.150045

20. Barding G.A. Jr., Fukao T., Beni S., et al. Differential metabolic regulation governed by the rice *SUB1A* gene during submergence stress and identification of alanylglycine by ^{1}H NMR spectroscopy // J Proteome Res. 2012. Vol. 11, No. 1. P. 320–330. DOI: 10.1021/pr200919b

21. Antonio C., Päpke C., Rocha M., et al. Regulation of primary metabolism in response to low oxygen availability as revealed by carbon and nitrogen isotope redistribution // Plant Physiol. 2016. Vol. 170, No. 1. P. 43–56. DOI: 10.1104/pp.15.00266

22. Herzog M., Fukao T., Winkel A., et al. Physiology, gene expression, and metabolome of two wheat cultivars with contrasting submergence tolerance // Plant Cell Environ. 2018. Vol. 41, No. 7. P. 1632–1644. DOI: 10.1111/pce.13211

23. Hasler-Sheetal H., Fragner L., Holmer M., Weckwerth W. Diurnal effects of anoxia on the metabolome of the seagrass *Zostera marina* // Metabolomics. 2015. Vol. 11, No. 5. P. 1208–1218. DOI: 10.1007/s11306-015-0776-9

24. Parveen M., Miyagi A., Kawai-Yamada M., et al. Metabolic and biochemical responses of *Potamogeton anguillanus* Koidz. (Potamogetonaceae) to low oxygen conditions // J. Plant Physiol. 2019. Vol. 232. P. 171–179. DOI: 10.1016/j.jplph.2018.11.023

25. Locke A.M., Barding G.A. Jr., Sathnur S., et al. Rice *SUB1A* constrains remodelling of the transcriptome and metabolome during submergence to facilitate post-submergence recovery // Plant Cell Environ. 2018. Vol. 41, No. 4. P. 721–736. DOI: 10.1111/pce.13094

26. Coutinho I.D., Henning L.M.M., Döpp S.A., et al. Identification of primary and secondary metabolites and transcriptome profile of soybean tissues during different stages of hypoxia // Data in Brief. 2018. Vol. 21. P. 1089–1100. DOI: 10.1016/j.dib.2018.09.122
27. theplantlist.org [Электронный ресурс]. The Plant List [дата обращения: 20.11.2022]. Доступ по ссылке: http://theplantlist.org/1.1/browse/A/Onagraceae/Epilobium/
28. mobot.org [Электронный ресурс]. Angiosperm phylogeny website, version 14 [дата обращения: 20.11.2022]. Доступ по ссылке: http://www.mobot.org/MOBOT/Research/APweb/orders/myrtalesweb2.htm#Onagraceae
29. Маевский П.Ф. Флора средней полосы европейской части России. 11-е изд. Москва: Товарищество научных изданий КМК, 2014. 635 с.
30. Ронжина Д.А. Экологическая дифференциация инвазионных и аборигенных видов рода *Epilobium* в прибрежно-водных экосистемах связана с функциональными особенностями растений // Российский журнал биологических инвазий. 2020. № 1. С. 38–51.
31. Чирков Ю.И. Основы агрометеорологии. Ленинград: Гидрометеоиздат, 1988. 248 с.
32. Puzanskiy R.K., Yemelyanov V.V., Shavarda A.L., et al. Age- and organ-specific differences of potato (*Solanum phureja*) plants metabolome // Russ J Plant Physiol. 2018. Vol. 65, No. 6. P. 813–823. DOI: 10.1134/S1021443718060122
33. Lai Z., Tsugawa H., Wohlgemuth G., et al. Identifying metabolites by integrating metabolome databases with mass spectrometry cheminformatics // Nat Methods. 2018. Vol. 15. P. 53–56. DOI: 10.1038/nmeth.4512
34. Hummel J., Selbig J., Walther D., Kopka J. The Golm Metabolome Database: a Database for GC-MS based metabolite profiling. Metabolomics. Vol. 18: Topics in Current Genetics / J. Nielsen, M.C. Jewett, editors. Berlin; Heidelberg: Springer. 2007. P. 75–95. DOI: 10.1007/4735_2007_0229
35. r-project.org [Электронный ресурс]. R Core Team. R: The R Project for Statistical Computing [дата обращения: 20.11.2022]. Доступ по ссылке: https://www.r-project.org/
36. CRAN.R-project.org [Электронный ресурс]. Komsta L. outliers: Tests for Outliers. R package version 0.15, 2022 [дата обращения: 20.11.2022]. Доступ по ссылке: https://CRAN.R-project.org/package=outliers
37. bioconductor.org [Электронный ресурс]. Hastie T., Tibshirani R., Narasimhan B., Chu G. Impute: Imputation for microarray data. R package version 1.70.0. 2022. Доступ по ссылке: https://bioconductor.org/packages/release/bioc/html/impute.html
38. Stacklies W., Redestig H., Scholz M., et al. pcaMethods — a Bioconductor package providing PCA methods for incomplete data // Bioinformatics. 2007. Vol. 23, No. 9. P. 1164–1167. DOI: 10.1093/bioinformatics/btm069
39. Thevenot E.A., Roux A., Xu Y., et al. Analysis of the human adult urinary metabolome variations with age, body mass index and gender by implementing a comprehensive workflow for univariate and OPLS statistical analyses // J Proteome Res. 2015. Vol. 14, No. 8. P. 3322–3335. DOI: 10.1021/acs.jproteome.5b00354
40. Brereton R.G., Lloyd G.R. Partial least squares discriminant analysis: taking the magic away // J Chemom. 2013. Vol. 28, No. 4. P. 213–225. DOI: 10.1002/cem.2609
41. Gu Z., Eils R., Schlesner M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data // Bioinformatics. 2016. Vol. 32, No. 18. P. 2847–2849. DOI: 10.1093/bioinformatics/btw313
42. Korotkevich G., Sukhov V., Sergushichev A. Fast gene set enrichment analysis // bioRxiv. 2019. P. 1–40. DOI: 10.1101/060012
43. Kanehisa M., Furumichi M., Sato Y., et al. KEGG for taxonomy-based analysis of pathways and genomes // Nucleic Acids Res. 2022. ID gkac963. DOI: 10.1093/nar/gkac963
44. bioconductor.org [Электронный ресурс]. Tenenbaum D., Maintainer B. KEGGREST: Client-side REST access to the Kyoto Encyclopedia of Genes and Genomes (KEGG). 2022. R package version 1.36.2. Доступ по ссылке: https://www.bioconductor.org/packages/release/bioc/html/KEGGREST.html
45. Shannon P., Markiel A., Ozier O., et al. Cytoscape: A software environment for integrated models of biomolecular interaction networks // Genome Res. 2003. Vol. 13, No. 11. P. 2498–2504. DOI: 10.1101/gr.1239303
46. Xu Y., Fu X. Reprogramming of plant central metabolism in response to abiotic stresses: A metabolomics view // Int J Mol Sci. 2022. Vol. 23, No. 10. ID5716. DOI: 10.3390/ijms23105716
47. Shingaki-Wells R.N., Huang S., Taylor N.L., et al. Differential molecular responses of rice and wheat coleoptiles to anoxia reveal novel metabolic adaptations in amino acid metabolism for tissue tolerance // Plant Physiol. 2011. Vol. 156, No. 4. P. 1706–1724. DOI: 10.1104/pp.111.175570
48. Mustroph A., Barding G.A. Jr., Kaiser K.A., et al. Characterization of distinct root and shoot responses to low-oxygen stress in Arabidopsis with a focus on primary C- and N-metabolism // Plant Cell Environ. 2014. Vol. 37, No. 10. P. 2366–2380. DOI: 10.1111/pce.12282
49. Fukushima A., Kuroha T., Nagai K., et al. Metabolite and phytohormone profiling illustrates metabolic reprogramming as an escape strategy of deepwater rice during partially submerged stress // Metabolites. 2020. Vol. 10, No. 2. ID 68. DOI: 10.3390/metabo10020068
50. Dacrema M., Sommella E., Santarcangelo C., et al. Metabolic profiling, *in vitro* bioaccessibility and *in vivo* bioavailability of a commercial bioactive *Epilobium angustifolium* L. extract // Biomed Pharmacother. 2020. Vol. 131. ID110670. DOI: 10.1016/j.biopha.2020.110670
51. Ak G., Zengin G., Mahomoodally M.F., et al. Shedding light into the connection between chemical components and biological effects of extracts from *Epilobium hirsutum*: Is it a potent source of bioactive agents from natural treasure? // Antioxidants. 2021. Vol. 10, No. 9. ID 1389. DOI: 10.3390/antiox10091389
52. Matysik J., Alia A., Bhalu B., Mohanty P. Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants // Curr Sci. 2002. Vol. 82, No. 5. P. 525–532.
53. Tamang B.G., Fukao T. Plant adaptation to multiple stresses during submergence and following desubmergence // Int J Mol Sci. 2015. Vol. 16, No. 12. P. 30164–30180. DOI: 10.3390/ijms161226226
54. Shikov A.E., Chirkova T.V., Yemelyanov V.V. Post-anoxia in plants: reasons, consequences, and possible mechanisms // Russ J Plant Physiol. 2020. Vol. 67, No. 1. P. 45–59. DOI: 10.1134/S1021443720010203
55. Yemelyanov V.V., Lastochkin V.V., Prikazyuk E.G., Chirkova T.V. Activities of catalase and peroxidase in wheat and rice plants under conditions of anoxia and post-anoxic aeration // Russ J Plant Physiol. 2022. Vol. 69, No. 6. P. 117. DOI: 10.1134/S1021443722060036
56. Shelp B.J., Bown A.W., McLean M.D. Metabolism and functions of gamma-aminobutyric acid // Trends Plant Sci. 1999. Vol. 4, No. 11. P. 446–452. DOI: 10.1016/S1360-1385(99)01486-7

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