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The effect of seasonal changes in mineral nutrition and secondary metabolism of *Epipactis palustris* (Orchidaceae) on the rhizosphere soil ecosystem

N. V. Zaimenko, N. S. Chernikova, N. P. Didyk, N. A. Pavliuchenko,
O. P. Yunosheva, I. P. Kharytonova, O. V. Zakrasov, A. M. Gnatiuk, M. B. Gaponenko

M. M. Gryshko National Botanical Garden of National Academy of Sciences of Ukraine, Kyiv, Ukraine

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M. M. Gryshko National Botanical Garden of National Academy of Sciences of Ukraine, Sadovo-Botanichna st., 1, Kyiv, 01103, Ukraine. Tel.: +38-050-660-57-95. E-mail: gnatiukalla@gmail.com

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Epipactis palustris (L.) Crantz is a rare orchid species whose ecological and biochemical strategies of adaptation remain insufficiently studied, despite its relevance for biodiversity conservation and ecosystem stability. This research aimed to investigate the seasonal changes in mineral nutrition, secondary metabolism, enzymatic activity, allelopathic potential, and microbial composition of the rhizosphere soil. Fieldwork was conducted in 2024 at the M. M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine (Kyiv). The study revealed that *E. palustris* demonstrates pronounced physiological and biochemical flexibility during the growing season, maintaining mineral balance even under conditions of reduced soil macro- and microelement availability. Elevated accumulation of Ca, Mg, Sr, and Si in the leaves indicated selective ion uptake and the development of ion-homeostatic mechanisms, which are crucial for structural stability and seasonal adaptation. Stable levels of brassinosteroids in the plants throughout the season reflected its ability to sustain hormonal–mineral regulation of growth, photosynthetic activity, and antioxidant defense. In summer, decreased laccase activity and humus content, combined with increased bicarbonate concentration and soil electrical conductivity, pointed to intensified transformation of organic matter and mobilization of mineral elements, creating favorable conditions for plant nutrition. The rhizosphere soil exhibited moderate allelopathic activity, supporting symbiotic interactions with microbial communities. Seasonal shifts in microbial complexes suggested dynamic pathways of organic matter turnover, while the consistent presence of nitrogen-fixing microorganisms ensured nitrogen supply. Taken together, these findings highlight the integrated role of mineral nutrition, hormonal regulation, and rhizosphere interactions in maintaining functional stability of *E. palustris*. The study provides new insights into the mechanisms of adaptation of rare orchids to environmental stress and emphasizes their connection with mycorrhizal associations. Beyond its ecological significance, this work contributes to the development of scientific approaches for *in situ* and *ex situ* conservation of rare orchid species, offering a framework for sustainable management of vulnerable plant populations under changing environmental conditions.

Keywords: rare plants; nutrient cycling; allelopathic activity; brassinosteroids; soil microbiome; plant adaptation.

Introduction

Under modern conditions of climate change and increasing anthropogenic pressure, research into the resilience of rare and endangered plant species is becoming particularly relevant (Zaimenko et al., 2024). Among them is marsh helleborine (*Epipactis palustris* (L.) Crantz.) – a representative of the orchid family (Orchidaceae Juss.), listed in the *Red Data Book of Ukraine* (Didukh, 2009) and Appendix II of CITES (CITES, 2025, https://checklist.cites.org/#/en). The species can be regarded as an indicator of ecosystem health due to its high dependence on soil moisture, nutrient availability, and biotic interactions within the rhizosphere.

It is for this reason that studying the seasonal dynamics of mineral nutrition, the content of secondary metabolites, and the characteristics of the soil microbiome under *E. palustris* plants allows for a deeper understanding of the ecological strategies of species at high risk of extinction in the wild and contributes to the development of effective measures for their protection.

Epipactis palustris is a perennial rhizomatous geophyte, distributed primarily across the temperate biome throughout Europe and the Middle East, extending its range eastward as far as Iran (POWO, 2026 https://powo.science.kew.org). It occurs mainly in alkaline soils of marshes, dune slacks, and similar wet areas (Lewis & Mielcarek, 2021). The range of marsh helleborine in Ukraine encompasses Polissia, the Carpathians, Roztochia, Opillia, the Forest-Steppe, the Steppe (mainly the valleys of large rivers), and the Mountainous Crimea (Didukh, 2009). However, its range is shrinking everywhere (especially in the plains) due to the disappearance of its biotopes (Mathé, 2015). *E. palustris* can develop in atypical ecological conditions for the species, indicating its high ecological plasticity. The orchid family is distinguished among vascular plants by the presence of vestigial seeds, which are most effectively dispersed by wind (Maalouf et al., 2024).

It is known that the distribution of representatives of the genus *Epipactis* is influenced by soil type and the nature of the bedrock (Pacsai et al., 2022). Marsh helleborine exhibits a certain plasticity regarding the substrate: Numerous locations attest to its ability to grow in soils formed on various geological carbonate rocks, as well as in serpentinite and siliceous soils. This demonstrates the plant's broad ecological specialization in relation to the soil-geological environment, although requirements for moisture and soil conditions remain decisive (Djordjević et al., 2022). In Ukraine, marsh helleborine prefers wet and damp peat-bog soils (Didukh, 2009).

It has been established that the rhizosphere of *E. palustris* is predominantly inhabited by representatives of Basidiomycota, mainly Tulasnellaceae and Ceratobasidiaceae, which act as key symbiotic partners for seed germination and plant nutrition, ensuring the plant's survival in specific wet ecotopes (Jacquemyn et al., 2016).

Kluza-Wieloch & Wyrzykiewicz-Raszewska (2016) identified a tendency for *E. palustris* plants to grow primarily in areas with high humidity; however, some subpopulations were found to be sensitive to anthropogenic impacts.

The main factors affecting the conservation of populations are land drainage, changes in agricultural practices, urbanization, the expansion of agricultural production, landscape and land-use transformation, the presence of drainage systems, overgrazing, or irrational meadow management (Kluza-Wieloch & Maciejewska-Rutkowska, 2015). Additional negative factors include mining and climate chan-

ge, which can lead to the drying out or loss of wetland ecosystems (Djordjević et al., 2022).

The literature data indicate that orchids use specific mechanisms to attract pollinators, including a certain type of scent and flower structure that is attractive to pollinating insects (Jacquemyn et al., 2014), and also color variations (Lewis & Mielcarek, 2021). In the study by Brantjes (1981), a semi-wild population of *E. palustris* was visited by honeybees (25%), sawflies, parasitic hymenopterans, ants (50%), bumblebees (20%), and other insects (1%). However, in urban conditions, dipterans (flies) and hymenopterans (bees, wasps) play a leading role in pollination. The specificity of interaction with pollinators in an urbanized environment has been noted, where the species composition of insects differs from that in natural biotopes; this confirms the species' ability to maintain a successful pollination system even under stressful urban conditions, which may be one of the factors of its stability and distribution (Gnatiuk et al., 2023).

The reproductive success of *E. palustris* largely depends on the quality and chemical composition of the nectar, not just flower morphology. The nectar of *E. palustris* contains a wide range of biologically active compounds (sugars, amino acids, and secondary metabolites) which can either attract or repel pollinating insects. It was found that individuals in natural populations produced nectar with a higher amount of sugars and amino acids; this highlights the role of biochemical mechanisms of interaction with pollinators in forming the adaptive strategies of *E. palustris* plants (Brzosko et al., 2021).

Populations of marsh helleborine are capable of growing even in highly transformed environments and are often characterized by morphological variability, indicating the high plasticity of the species. Vital indicators between natural and anthropogenic habitats exhibited differentiation: In urban conditions, certain traits may be suppressed, but generally, the plants retain their reproductive capacity. The species *E. palustris* demonstrates a significant ecological and morphological range of adaptation, allowing it to survive in the urban environment and serve as an indicator of ecosystem state and the adaptive potential of orchids in the context of urbanization (Kluza-Wieloch et al., 2017).

It is known that in the natural environment, the phenological phases of *E. palustris* usually occur later than under controlled conditions. In garden settings, plants begin flowering earlier and exhibit greater synchrony in phenological phases. Moreover, marsh helleborine grown in garden plots exhibited an extension of the entire growing season and a shortening of the spring phase of vegetative development. That is, *E. palustris* plants display phenological plasticity, and this factor influences reproductive success, survival, and distribution (Wyrzykiewicz-Raszewska, 2011).

It is a fact that a large number of *E. palustris* seeds have been found in early Holocene sediments; this discovey allows for the reconstruction of the distribution history and ecological conditions of this species' existence in the past (Gołaszewska et al., 2019). Paleobotanical analysis of peat bogs at current *E. palustris* sites showed that over the course of their history, vegetation composition of these ecosystems has underwent significant changes due to fluctuations in groundwater levels and changes in climatic conditions. The presence of *E. palustris* is associated with certain stages of development of wetland ecosystem, particularly with conditions of relative stability and sufficient moisture. Thus, the species can be considered an indicator of ecological balance in bog biotopes (Gałka & Kasper, 2011).

In modern literature, plant conservation is described through two key approaches: *in situ* (conservation in natural biotopes) and *ex situ* (outside their natural habitats). In the *in situ* context, the protection of *E. palustris* involves a range of measures: the preservation of natural habitats, which are most vulnerable due to land reclamation, intensive agriculture, and urbanization; the restriction of economic activities in areas where *E. palustris* grows; the creation of protected zones, sanctuaries, and nature reserves; systematic monitoring of populations for the timely detection of negative trends; and the ecological education of local communities aimed at preventing the destruction or unauthorized collection of plants (Gnatiuk et al., 2023; Ioniță & Jardan, 2023). *Ex situ* conservation involves vegetative and seed reproduction in laboratory conditions with the participation of symbiotic fungi, introduction into botanical gardens, and the creation of collections for long-term storage and possible restoration in the event of the disappearance of local populations (Gałka & Kasper, 2011; Gaponenko, 2013; Gołaszewska et al., 2019). A critical condition for protection is the maintenance of the hydrological regime: Without stable moisture, *E. palustris* populations degrade rapidly (Ioniță & Jardan, 2023).

It is known from literary sources that land management and the restoration of the water regime play a key role in the conservation of the species and significantly contribute to the appearance or recovery of orchid populations. *Epipactis palustrisis* was mentioned among those that have benefited from improved nature conservation management, especially in conditions of wet meadows or damp areas (Leten et al., 2022).

Modern botanists are investigating the features of distribution of marsh helleborine in natural and artificial conditions and are developing various measures for its protection. At the same time, the seasonal dynamics of mineral nutrition, the content of secondary metabolites (phenolic compounds, flavonoids, alkaloids), the influence of allelochemicals in the rhizosphere on the development of this species, and the role of mycorrhizal symbionts of *E. palustris* in plant nutrition and stress resistance remain poorly understood. The influence of marsh helleborine on the structure of plant communities and ecosystem functioning as a whole has also been insufficiently studied. Limited information exists regarding the species' response to climate change (drought, rising temperatures, groundwater level fluctuations). It has not been clarified which morphological and physiological-biochemical mechanisms ensure its survival under anthropogenic pressure. These areas define the relevance of further research on *E. palustris*.

The objective of this study was to elucidate the seasonal dynamics of mineral nutrition and secondary metabolism in *E. palustris* (Orchidaceae) and to assess their integrated effects on the rhizosphere soil ecosystem. Specifically, the research sought to quantify macro- and microelement composition in the plants and rhizospheric soil, evaluate the accumulation of secondary metabolites and the activity of laccase as indicators of biochemical adaptation, analyze allelopathic potential and microbial community structure in the rhizosphere, and determine how these factors collectively contribute to the functional stability and adaptive capacity of *E. palustris* under fluctuating environmental conditions. By addressing these objectives, the study aimed to provide novel insights into the mechanisms of stress tolerance and ecological resilience in rare orchid species, thereby informing strategies for their conservation both *in situ* and *ex situ*.

Materials and methods

This study was conducted in the M. M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine. The objects of the study were the rhizospheric soil and leaves of *E. palustris* (L.) Crantz plants growing on the collection plot of the Botanical Garden (Fig. 1). Sampling was conducted during the periods of *E. palustris* shoot development (May), flowering (July), and seed ripening (September) in 2024. A comparative analysis of the content of chemical elements in the soil and the aboveground part of the plants was performed, the functional structure of soil microbial communities was determined, and the allelopathic activity of the rhizospheric soil was evaluated.

The garden is located in the Pecherskyi District of Kyiv in southeastern part of city on the right bank of the Dnipro River (Fig. 1) in the temperate zone on the border of the forest and forest-steppe physiographic zones of Ukraine. The climate is moderately continental, with mild winters and warm summers (Vyshnevskyi et al., 2023). An analysis of weather data provided by the Meteopost (https://meteopost.com) online resource showed that the average annual temperature in Kyiv in 2024 was +12.2 °C, with total precipitation amounting to 420.4 mm. The distribution of precipitation during the observation year was uneven: In April and June, nearly 51% of the total annual precipitation occurred, while in May and September, it accounted for only approximately 0.1%. The gray forest soils with different textures dominate in the Botanical Garden. The artifici-

al population of *E. palustris* is located on the collection plot "Rare Species of Ukrainian Flora" with loamy-sandy soil.

The plants used in the research were cultivated and were not collected from natural habitats. All requirements of the Law of Ukraine on the *Red Book of Ukraine* were met. The study did not involve damaging or harvesting the plants.

Soil samples were collected with an auger at a depth of 10–20 cm and sieved through a 1–2 mm mesh. To determine soil pH, a portion of sieved dry soil was mixed with 25 mL of 1 N KCl solution (pH 5.5–6.0) and kept for 12 hours in a porcelain beaker. After 12 hours, measurements were taken using the pH/ORP meter HI 2211 (Hanna Instruments, Vöhringen, Germany, 2005).

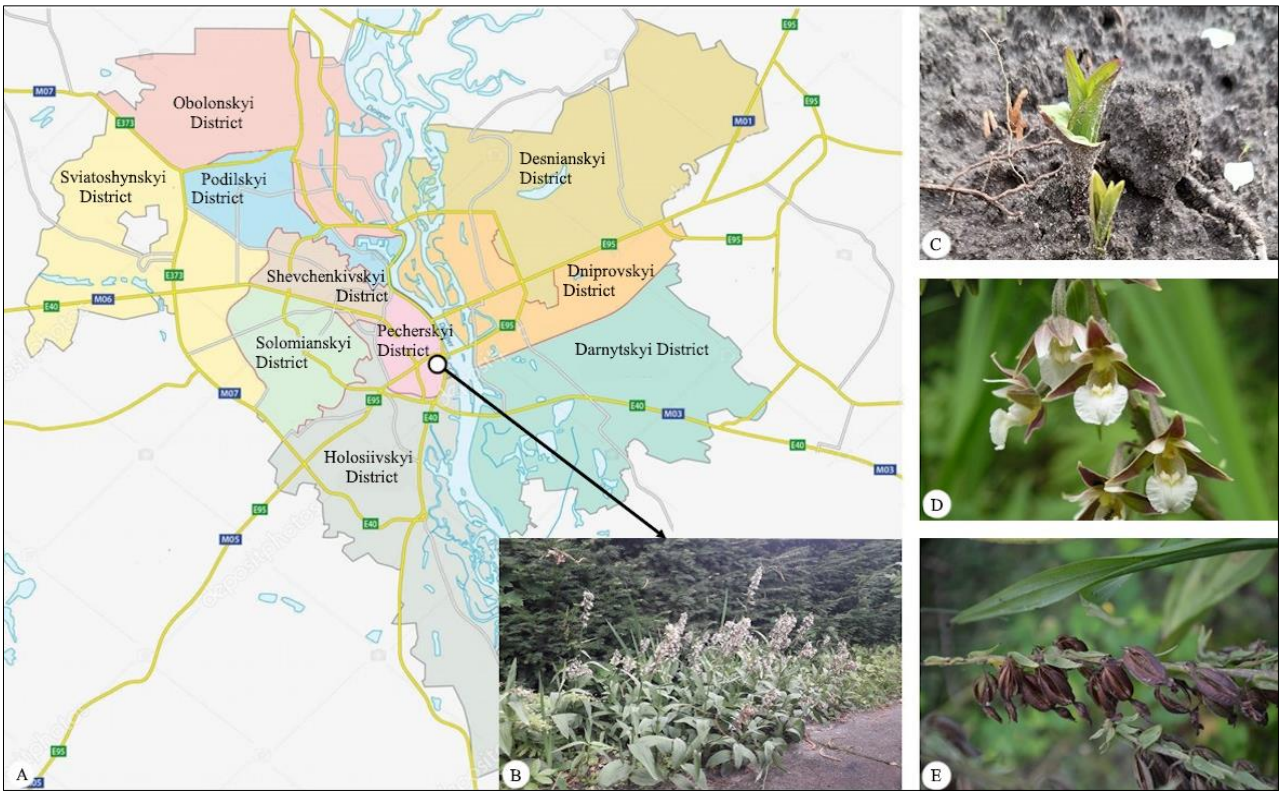

Fig. 1. *Epipactis palustris* (L.) Crantz in the M. M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine (Kyiv): *A* – the botanical garden on the Kyiv districts location map, *B* – plants growing on the collection plot, *C* – shoot development (May), *D* – flowering (July), *E* – seed ripening (September)

Sample preparation for determining macro- and microelement composition was carried out according to the method of Zaimenko (2021) by extracting acid-soluble forms of elements with 1.0 N nitric acid (HNO_3). Plant samples were pre-decomposed by wet ashing with a high-purity nitric acid solution using a SpeedwaveXpert DAP-60X microwave digestion system (Berghof GmbH, Eningen unter Achalm, Germany, 2018). The chemical element content was analyzed using an ICAP 6300 DUO plasma emission spectrometer (Thermo Fisher Scientific, Waltham, USA, 2008).

The limits of the relative uncertainty of the measurement results (U) of the mass fractions of the chemical elements in the samples did not exceed 20% (at k = 2, P = 0.95). The intralaboratory control of the accuracy of the measurement results was carried out using a certified standard moss sample M2 (Moss Reference Material M2, *Pleurozium schreberi* – The Finnish Forest Research Institute), using the compatibility criterion.

Allelopathic analysis of the soil was performed by direct bioassay using garden cress (*Lepidium sativum* L.) seedlings as a test object (Zaimenko, 2021).

Microbial composition analysis was performed by inoculating on solid selective media: micromycetes on Czapek's medium, actionmycetes on starch-ammonium agar (SAA), ammonifiers on meat-peptone agar (MPA), and microorganisms consuming mineral forms of nitrogen on SAA. Nitrogen-fixers were evaluated by the degree of soil aggregate colonization on Ashby's medium (Zaimenko, 2021). Microbiological studies were conducted on the day of sampling.

For the analysis of brassinosteroids (BS), extraction was performed in two stages. The first stage involved triple extraction with ethyl acetate (3 × 5 mL) from an aqueous leaf extract (1 g of tissue + 5 mL of extraction solution). The resulting ethyl acetate phase was evaporated *in vacuo*, and the residue was extracted with cyclohexane (5 mL). The second stage consisted of triple extraction with a mixture of ethanol and water (4:1, 3 × 5 mL) from the phase obtained after cyclohexane treatment. The ethanol extract was evaporated *in vacuo*, the residue was dissolved in a small volume of ethyl acetate, and the brassinosteroid content was then measured spectrophotometrically at a wavelength of 450 nm (Kravets et al., 2011).

To isolate flavonoids, 70% ethanol was used, and subsequent determination was carried out spectrophotometrically via the formation of a colored complex with a 10% solution of $AlCl_3$ (Zaimenko, 2021). Tannins were extracted with boiling distilled water. The quantitative content was determined by titration with 0.1% potassium permanganate solution in the presence of indigo carmine. Triterpenoids and saponins were extracted with 96% ethanol, and the quantitative content was determined spectrophotometrically using a color reaction with vanillin reagent (Zaimenko, 2021). Laccase activity was determined via the reaction with syringaldazine according to the method of Zaimenko (2021) using a SPECORD 200 instrument (Analytik, Jena, Germany, 2003).

All results are presented as the mean value ± standard error ($x \pm SE$). The significance of differences was assessed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test at a significance level of $P < 0.05$.

Results

The comparative analysis of the content of chemical elements in the soil and the marsh helleborine plants throughout the growing season (Tables 1, 2) indicated significant differences in the distribution of chemical elements. This suggests the possibility of using these data to

manage metabolic processes and the synthesis of biologically active compounds due to the plants' ability to regulate the uptake of macro- and microelements.

Table 1
Content of chemical elements in the soil under the *Epipactis palustris* plants throughout the growing season (mg/kg abs. dry matter, x ± SE, n = 5)

Element	Soil sampling period		
	May	July	September
Al	5189 ± 247a	2917 ± 139b	3294 ± 154b
B	4.97 ± 0.25a	4.16 ± 0.21a	0.098 ± 0.005b
Ba	61.3 ± 3.1a	53.7 ± 2.7a	32.8 ± 1.6b
Ca	3246 ± 163a	1928 ± 97b	2578 ± 129ab
Co	2.25 ± 0.12a	1.83 ± 0.09a	0.80 ± 0.05b
Cr	7.93 ± 0.41a	6.91 ± 0.37a	2.76 ± 0.14b
Cu	14.69 ± 0.75a	15.02 ± 0.77a	7.60 ± 0.37b
Fe	3927 ± 197a	2669 ± 134b	1844 ± 95c
K	1225.0 ± 61.3a	611.3 ± 31.5b	286.8 ± 14.8c
Mg	738.3 ± 37.1a	405.2 ± 19.5b	305.9 ± 15.4b
Mn	232.7 ± 11.4a	162.9 ± 8.1b	91.1 ± 4.7c
Na	244.5 ± 12.3a	34.7 ± 1.8b	247.1 ± 12.4a
Ni	4.42 ± 0.21a	4.46 ± 0.24a	2.95 ± 0.15b
P	4.04 ± 0.21a	8.24 ± 0.45b	9.04 ± 0.48b
S	519.6 ± 25.4a	448.2 ± 22.7b	834.2 ± 41.9c
Pb	25.73 ± 1.32a	24.94 ± 1.25a	20.04 ± 1.08b
Si	805.7 ± 41.2a	400.8 ± 21.9a	72.8 ± 3.7b
Sr	17.93 ± 0.91a	9.73 ± 0.47b	17.14 ± 0.89a
Ti	189.9 ± 9.74a	61.36 ± 3.08b	11.26 ± 0.57c
Zn	48.79 ± 2.49a	41.45 ± 2.08a	26.01 ± 1.32b

Note: different letters indicate values that reliably differ one from another within one line of the table according to the results of comparison using the Tukey test.

Table 2
Content of chemical elements in the leaves of the *Epipactis palustris* plants throughout the growing season (mg/kg abs. dry matter, x ± SE, n = 5)

Element	Plant sampling period		
	May	July	September
Al	463.7 ± 23.2a	210.5 ± 10.7b	407.8 ± 19.8a
B	17.20 ± 0.84 a	14.50 ± 0.76b	18.20 ± 0.97a
Ba	12.92 ± 0.69a	8.93 ± 0.51b	14.06 ± 0.69a
Ca	7591 ± 381a	6103 ± 307b	12880 ± 654c
Co	0.41 ± 0.03a	0.30 ± 0.02b	0.26 ± 0.01b
Cr	3.28 ± 0.18a	2.05 ± 0.11b	1.70 ± 0.09b
Cu	15.42 ± 0.81a	11.77 ± 0.60b	10.24 ± 0.54b
Fe	396.7 ± 19.7a	189.9 ± 9.8b	311.0 ± 16.2c
K	17830 ± 913a	8170 ± 421b	5915 ± 305c
Mg	1376 ± 70a	1095 ± 55b	1709 ± 86c
Mn	36.62 ± 1.92a	18.27 ± 0.95b	24.65 ± 1.25b
Na	70.9 ± 3.6a	43.1 ± 2.2b	152.6 ± 7.7c
Ni	3.64 ± 0.23a	2.78 ± 0.15b	1.90 ± 0.09c
P	23.09 ± 1.18a	32.53 ± 1.60b	20.21 ± 1.03a
S	2376 ± 121a	1248 ± 65b	2170 ± 109a
Pb	2.89 ± 0.15a	1.60 ± 0.09b	1.67 ± 0.08b
Si	791.9 ± 40.8a	321.9 ± 17.1b	578.7 ± 31.5c
Sr	23.72 ± 1.21a	20.43 ± 0.08a	67.82 ± 3.42b
Ti	17.11 ± 0.89a	3.82 ± 0.24b	10.85 ± 0.57c
Zn	55.7 ± 2.9a	31.8 ± 1.6b	34.4 ± 1.9b

Note: see Table 1.

The content of brassinosteroids (Table 3) in the *E. palustris* leaves remained relatively stable throughout the entire growing season, indicating the continuous maintenance of the regulatory balance necessary for the species' adaptation to wet biotope conditions. Their highest level in May may be associated with intensive shoot and leaf growth during the early vegetative phase, when brassinosteroids stimulate cell division and elongation. The decrease in their concentration by September corresponds to the transition to the stabilization phase and the completion of the vegetative cycle, when the need for active growth regulation decreases.

From May to September, the laccase activity decreased (Table 4). Laccase is the primary enzyme involved in the transformation of organic matter, the oxidation of phenolic compounds, and the formation of stable humus structures. Measurement of the pH of soil samples showed a slight change during the season within 6.4–6.8. The concentration of HCO_3^- (bicarbonates) increased from May to July, remaining elevated in September, which may be a result of more active microbial mineralization of organic matter and the release of carbon compounds during the summer period. An increased level of HCO_3^- contributes to the buffering properties of the soil, stabilizing the pH and creating favorable conditions for the growth of *E. palustris*.

Table 3
Content of brassinosteroids in the leaves of the *Epipactis palustris* plants throughout the growing season, ng/g of dry matter (x ± SE, n = 3)

May	July	September
0.69 ± 0.04a	0.65 ± 0.03a	0.62 ± 0.03a

Note: see Table 1.

Soil electrical conductivity increased significantly in July, compared with May, and then decreased slightly in September. This indicates an accumulation of soluble salts during the summer period, likely due to enhanced mineralization of organic matter and moisture evaporation. The decrease toward autumn may be the result of salt leaching by atmospheric precipitation and its absorption by the plant's root system.

Table 4
Humus content, laccase activity, and indicators of HCO_3 and electrical conductivity in the soil under the *Epipactis palustris* plants throughout the growing season (x ± SE, n = 3)

Indicator	Sampling period		
	May	July	September
Laccase activity, mV/g ± SE	105.1 ± 5.2a	98.6 ± 4.8a	90.3 ± 4.5b
Labile forms of humus, %	3.2	3.1	3.0
HCO_3, mEq/L H_2O ± SE	0.17 ± 0.08a	0.38 ± 0.01b	0.35 ± 0.02b
Electrical conductivity, µS/cm ± SE	109.0 ± 5.5a	185.0 ± 8.7b	142.0 ± 7.2c

Note. See Table 1.

A moderate allelopathic effect of the marsh helleborine was identified at all sampling periods, which may indicate the ecological strategy of orchids adapting to symbiotic relationships (mycorrhiza, interaction with microbiota). The inhibition of growth processes in acceptor plants under the influence of physiologically active compounds of the orchid ranged 42.5–52.2%. In the spring, a decrease in allelopathic activity was observed. During the summer, the allelopathic activity of the soil remained unchanged (Table 5). In the autumn, an increase in soil phytotoxicity was observed under the influence of the accumulation of exometabolites and the products of organic residue decomposition during the growing season.

Table 5
Allelopathic activity of soil by the direct bioassay method (biotest – root growth of *Lepidium sativum* L.), % of the control (x ± SE, n=5)

May	July	September
57.5 ± 1.7a	55.2 ± 1.6a	47.8 ± 1.4b

Note: see Table 1.

The obtained results are consistent with the evaluation of the rhizospheric soil microbiota, the abundance and species composition of which change significantly throughout the growing season (Table 6). The number of actinomycetes increased slightly from the flowering phase to the end of the growing season. During the flowering phase, the number of ammonifiers was higher than at the end of the growing season. The number of microorganisms consuming mineral nitrogen was also higher in the flowering phase than at the end of the growing season. In the flowering phase, the mineralization coefficient was also somewhat higher compared with the end of the growing season.

The growth of nitrogen-fixing microorganisms was assessed according to the percentage of soil aggregate colonization on Ashby's medium. In the flowering phase, the number of nitrogen-fixing microorganisms was 98%, and at the end of the growing season it accoun-

ted for 100%. Such dynamics indicate the relative stability of the nitrogen-fixing community in the rhizosphere of the marsh helleborine during the growing season, which ensures the plant is supplied with nitrogen throughout the entire developmental cycle, even during periods of decreased activity in other microbial groups.

Table 6
Number of taxonomic and functional groups of microorganisms in the soil under the studied plants of *Epipactis palustris* (x ± SE, n = 5)

Indicator	Sampling period		
	May	July	September
Micromycetes, thousand CFU*/g dry soil	13.7 ± 2.3a	22.6 ± 2.3b	15.3 ± 1.1a
Actinomycetes, million CFU*/g dry soil	0.5 ± 0.1a	0.6 ± 0.1b	0.9 ± 0.1c
Ammonifiers, million CFU*/g dry soil	6.7 ± 0.2a	7.0 ± 0.2a	5.5 ± 0.1b
Microorganisms consuming mineral nitrogen, million CFU*/g dry soil	5.0 ± 0.2a	5.3 ± 0.2a	3.9 ± 0.2b
Mineralization coefficient	0.7	0.8	0.7

Note: * CFU – colony-forming unit; different letters indicate values that reliably differ one from another within one column of the table according to the results of comparison using the Tukey test; the mineralization coefficient is calculated by the ratio of the abundance of microorganisms consuming mineral nitrogen (column 5) and ammonifires (column 4).

The analysis of the content of phenolic secondary metabolites in the *E. palustris* leaves revealed moderately high contents of flavonoids, tannins, and saponins and a moderate content of triterpenoids, as compared with other orchid species. At the same time, the concentration of the studied secondary metabolites in the leaves was characterized by the high seasonal amplitude of variation. The highest values of relative range of variation (RRV) were recorded for saponin and flavonoid contents (33% and 28%, respectively). Meanwhile, the triterpenoid content was relatively stable with the RRV = 11%. The pattern of the seasonal dynamics of the content of phenolic secondary metabolites (flavonoids and tannins) was similar: an increase from May to July and a decrease in September. At the same time, the contents of triterpenoids and saponins were the highest in May and gradually declined over the subsequent period (Table 7).

Table 7
Content of secondary metabolites in leaves and the rhizosphere soil of *Epipactis palustris* (mg/g DW, x ± SE, n = 4)

Secondary metabolites	Sampling period		
	May	July	September
Leaves			
Flavonoids	14.39 ± 0.14a	19.04 ± 0.13b	15.70 ± 0.08c
Tannins	7.26 ± 0.12a	9.12 ± 0.12b	8.29 ± 0.08c
Saponins	2.48 ± 0.11a	2.45 ± 0.14a	1.71 ± 0.06b
Triterpenoids	7.59 ± 0.09a	7.25 ± 0.09a	6.44 ± 0.09b
Rhizosphere soil			
Triterpenoids	0.19 ± 0.01a	0.18 ± 0.01a	0.16 ± 0.01b
Saponins	0.17 ± 0.02a	0.16 ± 0.02a	0.14 ± 0.01a

Note: see Table 1.

Additionally, saponins and triterpenoids were detected in the rhizosphere soil under *E. palustris*. Despite the moderate content of triterpenoids in the leaves of *E. palustris*, their concentration in the soil, as well as the concentration of saponins, was moderately high throughout the growing season. The seasonal dynamics of the accumulation of these secondary metabolites in the rhizosphere soil was characterized by a maximum in May, followed by a gradual decrease.

Discussion

Mineral nutrition is essential for normal growth and development, ensuring plant resistance to stress factors and protection against pathogens (Karthika et al., 2018; Tripathi et al., 2022; Hawkesford et al., 2023; Kirkby, 2023; Sharma et al., 2025). In our studies, a decrease in the concentrations of most macro- and microelements in the soil (Al, Fe, K, Mg, Mn, Si, Ti, Zn) was observed from May to September. This may be due to active uptake by *E. palustris* plants during the phase of intensive growth and a decrease in the availability of elements in the soil solution as a result of soil drying during summer. The increase in Ca, Na, P, S, and Sr in the soil by the end of the growing season indicates a redistribution and accumulation of these elements in the soil-plant system, which is important for maintaining the physiological and biochemical stability of orchids. The detection of Cd in the soil only in September may be a consequence of heavy metal mobilization, changes in soil pH and moisture, and reflects seasonal fluctuations in the bioavailability of toxicants.

The orchid leaves were characterized by a high accumulation of Ca, K, and Mg, which is consistent with typical plant requirements for maintaining ionic homeostasis and cell wall stability. The highest Ca content in September indicates a reduction in membrane stress, maintenance of soil balance, and adaptation to stresses at the end of the growing season (Pandey, 2018; Sarwar et al., 2019). The K content in the plant decreased sharply from May to September, indicating its intensive use in photosynthetic and osmotic processes during active growth (Xu et al., 2020). The amounts of Na and Sr in the plant material increased significantly in September, which may indicate the involvement of these elements in osmoregulation and ionic balance mechanisms under changing environmental moisture conditions. The increase in strontium levels in the leaves may also be related to an increase in nitrogen supply due to the active development of nitrogen-fixing microorganisms, as confirmed by the results of assessment of the microbiological soil activity. The elevated Si content in September suggests an enhancement of the barrier function of cell walls and improved mechanical strength in plants, confirming the presence of labile mechanisms enabling adaptation to altered environmental conditions. Furthermore, silicon improves the uptake of macro- and microelements, facilitates their assimilation by plants, and reduces their toxicity, helping plants utilize available nutrients more efficiently. In addition, it improves their physiological state, supports homeostasis, and mitigates the negative effects of stress on metabolism (Greger et al., 2018; Pavlovic et al., 2021).

Fluctuations in P and S throughout the season indicate their participation in the regulation of metabolic processes that also contribute to stress protection (Hawkesford, 2023).

The decrease in the concentrations of most elements in the soil from May to September was accompanied by increased or stable levels of Ca, Mg, Sr, and Si in the leaves, suggesting active selective absorption by the plant even with reduced availability in the soil. This confirms the high physiological and biochemical plasticity of *E. palustris* plants, which are able to maintain the necessary mineral balance and ensure functional stability in varying environmental conditions.

The decrease in laccase activity and humus content against the background of increasing HCO_3^- and electrical conductivity in summer reflects active processes of organic matter transformation and mobilization of mineral compounds. This creates conditions for providing plants with necessary nutrients and maintaining their functional stability throughout the growing season. The decrease in soil allelopathic activity in spring is associated with a reduction in the biochemical activity of plants and the gradual degradation of allelopathically active substances in the soil (Scavo et al., 2019). In the flowering phase, the number of micromycetes was higher compared with the end of the vegetation period, which is related to the period of the plant's most intensive metabolism, when more organic substances (exometabolites, root secretions) enter the soil. The decrease in fungal abundance at the end of the growing season may have been caused by the depletion of available substrates and seasonal inhibition of their activity. The number of actinomycetes increased slightly from the flowering phase to the end of the growing season. This can be explained by the fact that actinomycetes develop better on organic residues that accumulate throughout the season. They are known for their ability to decompose complex polymers and synthesize antibiotic substances capable of regulating the structure of the microbial community.

During the flowering phase, the number of ammonifiers was higher than at the end of the growing season, which is consistent with

the active process of organic matter decomposition during the period of intensive plant vital activity. A decrease in the number of ammonifiers at the end of the growing season may indicate a decline in the rates of organic nitrogen mineralization. The number of microorganisms consuming mineral nitrogen was also higher in the flowering phase than at the end of the growing season. This means that during the period of active growth and flowering, there is an intensive exchange of nitrogen compounds in the soil: The release of organic substances stimulates the development of microbiota, including nitrogen consumers.

In the flowering phase, the mineralization coefficient was slightly higher compared with the end of the growing season, indicating more active processes of nitrogen transformation in the soil during this period, which is consistent with the data on ammonifiers and nitrogen-consuming microorganisms.

Thus, *E. palustris* forms microbial complexes in the rhizosphere that change according to the phenological phases and are directed toward the synthesis of organic matter. In the flowering phase, the development of micromycetes, ammonifiers, and nitrogen consumers prevailed, leading to a higher intensity of mineralization and nitrogen exchange processes. At the end of the growing season, the abundance of these groups decreased, while the proportion of actinomycetes grew, indicating a transition of microbial processes to a slower phase of organic matter transformation. Maintaining a high level of nitrogen-fixing activity helps maintain the fertility of the rhizospheric soil and the formation of a favorable microbial environment. Furthermore, this stability is explained by the plant's active secretion of specific secondary metabolites that stimulate the development of nitrogen-fixing microorganisms. This underscores the important role of nitrogen fixation in ensuring the plant's resistance to soil-ecological fluctuations and indicates the ability of *E. palustris* to form stable microbial associations.

The leaves of *E. palustris* had a relatively high content of flavonoids, tannins, and saponins in comparison with other species of the orchid family (Hürkan et al., 2019; Natta et al., 2022). This indicates the important role of these secondary metabolites in the processes of adaptation of *E. palustris* to the environment. High amplitude of seasonal variation in foliar contents of saponin and flavonoid confirmed their important role in physiological reactions of *E. palustris* to meteorological conditions, such as elevated temperature, UV-PAR, and frequent droughts during the period from May to July of 2024. September of 2024 was characterized by milder weather conditions and the level of all studied secondary metabolites in *E. palustris* leaves was significantly lower.

An important feature of flavonoids is their ability to absorb light in the short-wave part of the spectrum (280–320 and 270–280 nm, respectively); therefore, one of the primary functions of these secondary metabolites is the protection of plant tissues from ultraviolet radiation. In addition, flavonoids and tannins function as signaling substances in their interactions with pathogenic and nitrogen-fixing microorganisms, protecting plants from phytophages and phytopathogens (Chechui, 2021; Melnychuk et al., 2022; Tonga et al., 2022). Phenolic compounds (phenolic acids, flavonoids, tannins, etc.) play an important role in the processes of plant adaptation to biotic and abiotic stress factors. Due to their high antioxidant activity, phenolic secondary metabolites neutralize the action of free radicals formed during oxidative stress and are capable of quenching the oxidative "burst". Phenolic compounds with an o-diphenolic structure are able to form chelates with metal ions, contributing to their detoxification and removal from the plant organism (Melnychuk et al., 2022). Phenolic secondary metabolites participate in respiration, photosynthesis, and the regulation of phytohormone synthesis, promoting the development of the root system and the intake of mineral substances into plants (Melnychuk et al., 2022).

Triterpenoids and saponins play an important role in protecting plants from phytopathogens and herbivores, regulating the process of fruit set and ripening by influencing the mechanical stability of the fruit surface (Dashbaldan et al., 2021). In addition, triterpenoids and saponins are signaling molecules participating in the communication between plants and associated soil microbiota (Didyk & Zaimenko, 2025). We found a high content of these secondary metabolites in the rhizosphere soil of *E. palustris*, which cannot be explained solely by the excretory function of the roots of *E. palustris*, considering their insignificant phytomass in the soil. Aerial parts of *E. palustris* also cannot have a significant effect on biochemistry due to the same reason. Apparently, the accumulation of saponins and triterpenoids in the soil is related to soil microbiota. Both endophytes and free-living bacteria and fungi are known for their potential to synthesize triterpenoids and saponins, which regulate their interactions with higher plants and other microorganisms (Jin et al., 2017; Didyk & Zaimenko, 2025). Ricci et al. (2014) established a significant positive correlation between the ability of bacteria and fungi to synthesize triterpenoids and their association with the rhizosphere of higher plants.

Our results regarding the content of allelochemicals in the foliar tissues of *E. palustris* indicate the high inherent resistance of this plant to nonspecific biotic and abiotic stresses. Allelochemicals enter the rhizosphere soil through precipitation, leaf leachates, root exudates, and litter decomposition products. They affect the soil microbiome, serving as a carbon source for some microorganisms while inhibiting others (Bao et al., 2022; Chen et al., 2024). Furthermore, allelochemicals influence the pools and fluxes of mineral nutrients in the soil (Hättenschwiler & Vitousek, 2000). Specifically, it has been established that phenolics can inhibit the processes of nitrification, as well as ammonification and the decomposition of soil organic matter (Hättenschwiler & Vitousek, 2000; Schmidt et al., 2013; Zaimenko et al., 2021), and may inhibit or stimulate symbiotic nitrogen-fixing (N_2) bacteria (Hättenschwiler & Vitousek, 2000). Numerous scientific evidences indicate the ability of polyphenols to inhibit the development of soil bacteria and stimulate fungi and actinomycetes (Zaimenko et al., 2021). Triterpenoids stimulate the development of symbiotic and free-living nitrogen-fixing bacteria, regulating the symbiotic relationships between these microorganisms and plants (Kulkarni et al., 2015).

Brassinosteroids are known as universal regulators of growth and resilience, participating in the activation of photosynthesis, antioxidant defense, and increasing plant tolerance to stresses (heat, salt, water deficit) (Chechui, 2021; Alnusairi et al., 2024). Given the results of the macro- and microelement assessment (Tables 1 and 2), it can be assumed that maintaining a stable level of brassinosteroids is linked to the ionic balance of Ca, Mg, and Si and their participation in signaling pathways. This indicates that *E. palustris* plants integrate hormonal and mineral regulation to ensure resilience to fluctuations in soil conditions throughout the season.

The decrease in laccase activity may be associated with soil depletion (phenols, lignin) throughout the season, as well as seasonal fluctuations in temperature and moisture affecting microbial activity in the rhizosphere (Hu et al., 2018). The content of labile forms of humus remains relatively stable with a slight decrease toward the end of the growing season, indicating a relative balance between the processes of mineralization and humification, characteristic of wet biotopes. The slight decrease toward the end of the growing season suggests the gradual use of organic resources in the vital processes of both the microbiota and the plants themselves. The data obtained prove that dynamic seasonal changes in soil biochemistry occur in the rhizosphere of the marsh helleborine.

The study was limited to a single growing season and conducted on one population cultivated under moderate care (including artificial irrigation during plant vegetation). Factors such as the mineral composition and pH of precipitation and irrigation water, biochemical interactions, and allelopathic effects of nearby plants (particularly trees) were not assessed. This constrains the extrapolation of the results to other populations, habitats, or climatic conditions. Future research should take these factors into account for a more comprehensive evaluation of adaptive mechanisms.

Conclusions

The research results have established the seasonal characteristics of mineral nutrition, secondary metabolism, allelopathic activity, and microbiological processes in the rhizospheric soil of *E. palustris* plants. These plants have a high adaptive potential due to a combinati-

on of biochemical, physiological, and microbiological mechanisms that ensure the preservation of the species in both natural and artificial biotopes.

Epipactis palustris plants exhibit high physiological and biochemical plasticity, manifested in their ability to maintain mineral balance even under conditions of decreasing content of most macro- and microelements in the soil throughout the growing season. The dynamics of brassinosteroid content in the leaves indicate the maintenance of hormonal-mineral regulation of growth, which ensures the optimization of photosynthetic activity, antioxidant protection, and adaptation to fluctuations in environmental conditions. Marsh helleborine has a specialized ecological strategy aimed at symbiotic relationships with microbiota and mycorrhizal organisms.

A review of the literary data and current research results allows for an assessment of the functional role of biochemical and microbiological factors in maintaining the viability of *E. palustris*. In the context of climate change and anthropogenic influence, the study of plant resistance mechanisms contributes to the understanding of survival strategies for rare and endangered orchid species. Of particular importance is the determination of the role of silicon in the formation of the adaptive potential of plants, which had not been systematically studied for this species previously.

For the first time, the features of mineral nutrition, microbiota activity, and secondary metabolism of *E. palustris* have been established, allowing for the optimization of conditions for the conservation of the species in natural and artificial biotopes. A moderate allelopathic influence of the marsh helleborine's rhizospheric soil was shown, which may indicate the ecological strategy of orchids adapting to symbiotic relationships. These results can be used to create effective programs for the introduction and reintroduction of orchids in botanical gardens and protected areas.

Determining the role of mycorrhizal fungi and the structure of the rhizosphere microbiota helps develop biotechnological approaches for symbiotic seed germination, which is critical for the *ex situ* reproduction of *E. palustris*. Establishing optimal soil conditions, moisture levels, acidity, and the supply of macro- and microelements can be used for the restoration of drained or degraded lands. Microbiological data can assist in the formation of soil microorganism consortia capable of improving rhizosphere structure and stimulating the growth of rare plants, while the biochemical indicators of the plants will serve as markers of the species' adaptive potential to climate change.

Thus, the results of the study create a foundation for modeling adaptation mechanisms in orchids to climatic stresses and for developing effective approaches to their protection and the restoration of natural populations in the context of modern ecological challenges, which requires further investigation.

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