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Adaptive Redox Reactions Promote Naturalization of Rare Orchid *Epipactis atrorubens* on Serpentine Dumps Post Asbestos Mining

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Abstract: *Epipactis atrorubens* (Hoffm.) Besser. is a regionally rare orchid species with highly ornamental properties due to its very beautiful bright flowers, therefore it is of considerable interest as a horticultural plant for use in botanical gardens and greenhouses. The objective of the research was to assess metal accumulation and some pro- and antioxidant reactions in *E. atrorubens*, colonizing serpentine dumps post asbestos mining. Additionally, some physicochemical properties of substrates, microbiotic characteristics and water status were investigated in orchids growing on two serpentine dumps and in a natural forest habitat of the Middle Urals, Russia. The dump substrates were characterized by the strong stoniness and the high content of Mg, Ni, Cr and Co (by 1.8 times on average) compared to the natural habitat. In these sites, *E. atrorubens* was characterized by increased mycorrhization. In the rhizome and roots of *E. atrorubens* the concentrations of most metals studied were considerably higher (more than 4 times on average) than in the leaves. It was found that orchids colonizing serpentine dumps produced more lipid peroxidation products (by 1.4 times on average) in the leaves which was accompanied by the more active synthesis of such non-enzymatic antioxidants as ascorbate, free proline, soluble phenolic compounds (including flavonoids) and non-protein thiols. The study suggests that non-enzymatic antioxidants increased the adaptive potential of *E. atrorubens* and contributed to its naturalization on serpentine dumps post asbestos mining.

Keywords: Orchidaceae; ornamental plant introduction; serpentine outcrops; stressful conditions; adaptive responses; plant water status; redox balance; non-enzymatic antioxidants

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1. Introduction

At present, the problem of preserving biological diversity is becoming increasingly important. Changes in natural habitats have lead to the extinction of many species, including a number of orchid plants [1]. At the same time, in recent decades, some species of rare orchids were found in anthropogenically disturbed habitats, including industrial dumps [2–9]. One of the representatives of the Orchidaceae family found in disturbed habitats in Russia is *Epipactis atrorubens* (Hoffm.) Besser. This species is listed on the European Red List of Vascular Plants under the category of 'Least Concern' [10] and is included in the Convention on International Trade in Endangered Species Protection Status (CITIS) [11]. It is also listed in the Red Book of many Russian regions, including the Sverdlovsk region under category III, 'Rare plant' [12].

Epipactis atrorubens is a short-rhizome herbaceous perennial, calcephilus, xeromesophyte that grows in dry and well-lit habitats [5]. This species is widely distributed in boreal,

temperate and submeridional zones. It is usually found in deciduous, coniferous and mixed forests [5,13,14]. This orchid has highly ornamental properties due to its beautiful bright flowers (Figure 1a,b). Therefore, it is interesting as a horticultural plant for growing in botanical gardens and greenhouses.

(**a**) (**b**)

Figure 1. *Epipactis atrorubens* on serpentine dump: (**a**) Flowering plant; (**b**) Orchid flower.

The conservation of rare plant species is often preceded by the study of their adaptive abilities under natural conditions. In this regard, it is necessary to have a complete understanding of the orchid's adaptive reactions that increase their resistance to stressful conditions. It is well known that *E. atrorubens* has a great colonization potential on different industrial dumps [2,4,15]. It was found also on the dumps of serpentine rocks formed during the development of asbestos deposits in the Middle Urals [6,7].

Serpentine substrates are commonly unfavorable for plant growth due to their negative physicochemical properties [16–19]. It is known that under the impact of the unfavorable effect of environmental factors, the number of reactive oxygen species (ROS) in plant cells can increase, which leads to the activation of prooxidant processes and the development of oxidative stress [20,21]. The accumulation of malondialdehyde (MDA) and other products of lipid peroxidation can be on the one hand an indicator reaction reflecting the degree of stress exposure [22] and, on the other hand, a signal for the gene expression of some antioxidant enzymes and non-enzymatic antioxidants [23,24]. In higher plants among non-enzymatic antioxidants, ascorbate and glutathione are the most abundant soluble forms which play a vital role as electron donors and scavenge ROS directly through the glutathione–ascorbate cycle [21]. Other antioxidants play an equally important role. For example, many phenolic compounds, including flavonoids, have a great potential to

scavenge free radicals and reduce cell damage from lipid peroxidation [21,25,26]. Free proline has a multifunctional effect, it plays an important role in both osmoregulation and antioxidant protection, providing cellular homeostasis and facilitating plant adaptation to stressful conditions [27]. In this regard, studies of plant antioxidant status and methods of stress mitigation are becoming increasingly important.

To date, some anatomical and morphological features, chemical composition, mycorrhizal associations and bacterial microflora of several representatives of the genus *Epipactis*, growing in natural and transformed ecosystems have been wellstudied [2,4,6,7,28–31]. However, the adaptive redox reactions of orchids growing abundantly on serpentine outcrops, have not been practically explored.

The aim of the research was to study metal accumulation and to assess some pro- and antioxidant reactions in *E. atrorubens*, colonizing serpentine dumps post asbestos mining. Additionally, the physicochemical properties of the rhizosphere substrate, rhizospheric microbiota and water status of this orchid were studied. The comparative analysis of *E. atrorubens*, growing in transformed and natural habitats will make it possible to identify the adaptive responses for stress mitigation contributing to rare orchid species naturalization and conservation.

2. Materials and Methods

2.1. Study Area

The study area is located within the Tagilo-Nevyansk hyperbasite massif on the eastern slope of the Middle Urals (Sverdlovsk region, Russia), belonging to the taiga zone, southern taiga subzone. Three naturally colonized plant populations of *E. atrorubens* were selected for the present study (Figure 2).

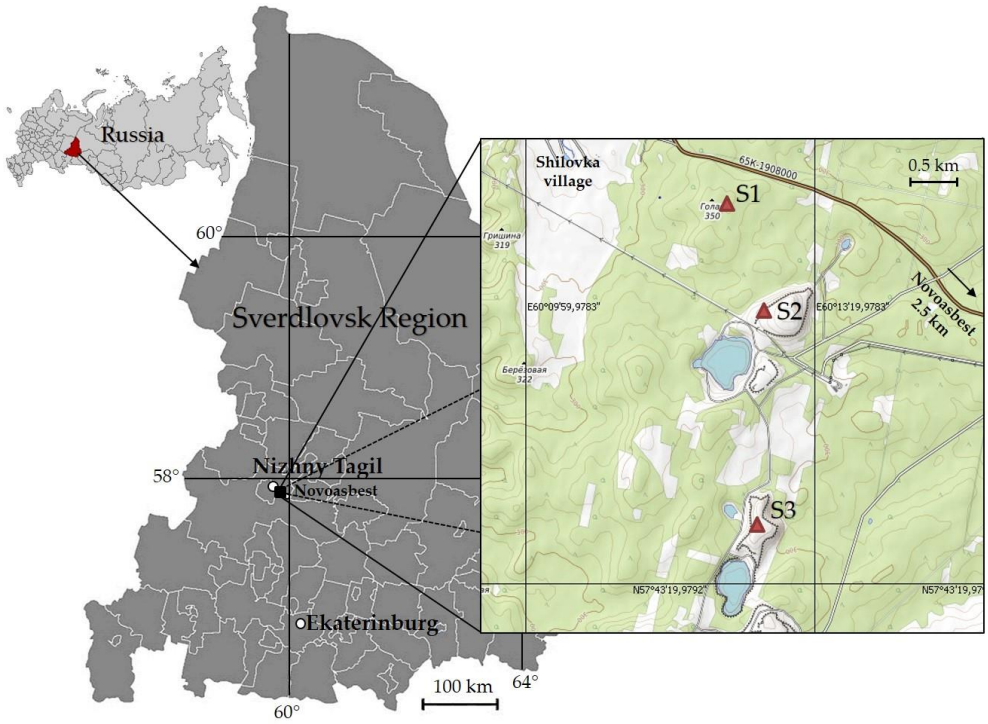

Figure 2. The studied sites of *E. atrorubens* populations (S1–S3) in the Sverdlovsk region, Russia.

The study area climate is characterized by a continental subarctic climate with an average annual air temperature of +1.0 °C; the average July temperature is +17.2 °C and the average January temperature is −16.0 °C (according to the weather station in Nizhny Tagil) [32]. The average annual rainfall is 628 mm. The snow cover lasts from the second

half of November to early April, and its average thickness is 0.7 m. The depth of soil freezing is 0.8–1.7 m.

As a reference control (S1), the population of *E. atrorubens* in a pine forest on the Golaya Mount slope, located between Shilovka and Novoasbest villages, about 25 km from Nizhny Tagil, Sverdlovsk region was chosen (Figure 2, Table 1).

Table 1. Studied sites for sampling of rhizospheric substrate and *E. atrorubens* plants.

Sites	Study Area	Coordinate	Plant Community
S1	Forest area on serpentine rocks	57°45′42.93″ N 60°12′52.82″ E	Natural pine forest community on the Golaya Mount slope
S2	Shilovsky dump of serpentine rocks of the Anatol'sko-Shilovsky asbestos deposit	57°44′54.78″ N 60°12′37.55″ E	Emerging pine forest community with sparse undergrowth of trees and shrubs on the lower tier
S3	Anatol'sky dump of serpentine rocks of the Anatol'sko-Shilovsky asbestos deposit	57°43′32.92″ N 60°12′39.44″ E	Pine forest community on the second tier ledge and gentle slope

Two other populations studied were found at serpentine dumps (S2 and S3) of the Anatol'sko-Shilovsky deposit, located about 2.0–4.0 km from S1 (Table 1). The area is confined to lenticular deposits of talc-chlorite-carbonate rocks and has been exploited for the extraction of fibrous asbestos ($Na_3(Mg,Fe^{2+})4Fe^{3+}Si_8O_{22}(OH)_2$) as an open cast mine from 1952 to 1992 [7]. Site S2 was represented by an emerging forest community located on the berm of the first tier on the southern side of the Shilovsky dump, while S3 was represented by a forest community formed on the eastern side of the Anatol'sky dump (Table 1). The total estimation of the forest communities formed on the study sites was carried out according to the generally accepted geobotanical methods [7].

The forest communities formed in the natural habitat (S1) and on the Anatol'sko-Shilovsky asbestos deposit (S2 and S3) showed uneven formation of vegetation. In reference site (S1) the average age of the stand of *Pinus sylvestris* L. was 70 years and the tree crown density was 0.6. The range of afternoon light intensity measured at the sampling point varied between 6 and 15 klx. Besides the dominant *P. sylvestris*, other species such as *Larix sibirica* Ledeb., *Betula pendula* Roth and *Picea obovata* Ledeb. were rarely found in the stand; sparsely isolated individuals of *Juniperus communis* L., *Rosa acicularis* Lindl., *Sorbus aucuparia* L. and *Chamaecytisus ruthenicus* (Fisch. ex Wol.) Klásk. grew in the undergrowth. *Vaccinium myrtillus* L., *V. vitis-idaea* L. and *Linnaea borealis* L. were represented in the herb-shrub layer. Herbaceous species were dominated by *Calamagrostis arundinacea* (L.) Roth, *Brachypodium pinnatum* (L.) Beauv., *Geranium sylvaticum* L., *Rubus saxatilis* L., *Fragaria vesca* L., *Potentilla erecta* (L.) Raeusch., etc. The total projective cover of the herb-shrub layer varied from 40 to 80%. *Epipactis atrorubens* occurred in groups of 3 to 14 individuals. The total number of orchid plants was about 43, the average density was about 8 individuals per 100 m^2. Flowering plants dominated the age spectrum (90%). The local population's self-maintenance was ensured through seed and vegetative propagation.

Site S2 was represented by an emerging forest community located on the berm of the first tier on the southern side of the Shilovsky dump. There, on a flat surface, rolled by road transport, there was a sparse undergrowth of *B. pendula* and *P. sylvestris* as well as some species of the *Populus* and *Salix* genera. There was no crown closure in the forest area and the afternoon light intensity in the sampling point was very high (varying between 80 and 105 klx). The herb-shrub layer was dominated by *Dendranthema zawadskii* (Herbich) Tzvel., *E. atrorubens*, *Thymus talijevii* Klok. & Des.-Shost., *Solidago virgaurea* L. and *Calamagrostis epigeios* (L.) Roth. The total projective cover of this layer varied from 0 to 15%. The distribution of *E. atrorubens* was uneven and the orchids grew along the roads, between stones and under the trees. The number of *E. atrorubens* in the local population was 189; the average density was about 32 individuals per 100 m^2. The age spectrum was dominated by pregenerative plants (58%).

Site S3 was represented by a forest community formed on the eastern side of the Anatol'sky dump. The tree layer was dominated by *P. sylvestris*. The age of the trees was between 10 and 35 years; the tree crown density was 0.5–0.6. The range of light intensity measured at the sampling point varied between 48 and 56 klx. *Calamagrostis arundinacea* dominated in the herb-shrub layer. The total projective cover of this layer varied from 0 to 20%. *Epipactis atrorubens* plants were found both as individuals and in groups of up to three individuals. The number of *E. atrorubens* in the local population was 163; the average density was about 33 individuals per 100 m^2. Individuals of the pregenerative age state prevailed (65%).

2.2. Collecting and Preparation of Plants and Substrates

Both plants and rhizospheric substrate were collected from studied habitats of *E. atrorubens* (S1–S3) over a two-year period (mid-July 2019 and 2020). All individuals were collected at the same phenological state (a fully developed inflorescence) under similar weather conditions (temperature during the daytime was about 24 ± 3 °C and the relative humidity was about 60 ± 5%).

Four generative orchid plants with 3–5 individual inflorescences (40–50 cm in length) were randomly selected from each site. The plants were carefully dug up together with the underground organs (rhizome + roots) and part of the soil (up to 15 cm in depth), placed in sterile plastic bags and transferred to the laboratory. The plant samples were cleaned of soil particles and washed first with running tap water, then with distilled and deionized water. One part of the fresh plant material (leaves and rhizome with roots) was fixed at 105 °C for 2 h and dried at 75 °C for 24 h for further metal analysis. The other part of the plant material (weighted fresh leaf cuttings) was partly used for the immediate determination of the *E. atrorubens* water status and partly frozen in liquid nitrogen and stored at −80 °C for further biochemical analysis. For the estimation of dry weight (DW), weighted fresh leaves were dried in a hot air oven at 75 °C for 24 h and the ratio of FW/DW was calculated.

The substrate samples were collected close to the orchid root zone from each studied site. The samples were air-dried for five days, oven-dried at 75 °C for 24 h and then used for granulometric and physicochemical analyses. Independently, part of the rhizospheric substrate from each site was used to determine some microbiological characteristics.

2.3. Physicochemical Characterization of Substrates

Part of the substrate was used for the determination of the percentage of different particle sizes which was performed by a standard sieve analysis (stones: >10 mm; gravel large: 5–10 mm; gravel small: 2–5 mm; sand large: 1–2 mm; sand average: 0.25–1 mm; dust and clay: <0.25 mm) as was described previously [7]. The second part of the soil was destoned, homogenized and passed through a sieve (<2 mm) [33] and a composite sample for each site was used to determine pH, electrical conductivity (EC) and available macronutrients (nitrogen, phosphorus, potassium), as well as total and available metal content.

The pH and EC of the substrate–water suspensions (1:2.5; *w*/*v*) were measured using a portable multivariable analyzer HI98129 Combo (Hanna Instruments GmbH, Graz, Austria). The alkaline-hydrolyzed nitrogen content and the available forms of phosphorus were measured as described by Filimonova et al. [7]. Subsequently, dried plant material (leaves and rhizome + roots) and soil samples were weighed and digested with concentrated nitric acid (analytical grade) using MARS 5 Digestion Microwave System (CEM, Matthews, NC, USA) for the determination of total metal concentration.

The substrate moisture content was determined using the thermostatic weight method [34] and expressed as a percentage of DW of the substrate.

The available form of metals was analyzed after mixing the substrate samples with 0.4 mM Na_2EDTA [35]. All the samples were prepared using double deionized Millipore water (Milli-Q system, Millipore, Molsheim, France). The Mg, Ca, K, Fe, Zn, Cu, Mn, Ni, Cr, Pb and Co concentrations in all samples were determined using a flame atomic absorption spectrometer AA240FS (Varian Australia Pty Ltd., Mulgrave, Victoria, Australia). Standard

Reference Materials [JSC Ural Plant of Chemical Reagents, Russia; GSS 7681-99 for Mg(II), GSS 7682-99 for Ca(II), GSS 8092-94 for K(I), GSS 7766-2000 for Fe(III), GSS 7256-96 for Zn(II), GSS 7998-93 for Cu(II), GSS 7266-96 for Mn(II), GSS 7265-96 for Ni(II), GSS 8035-94 for Cr(VI), GSS 7012-93 for Pb(II), and GSS 8089-94 for Co(II)] were used for the preparation and calibration of each analytical batch. Calibration coefficients were maintained at a high level of not less than 0.99.

2.4. Assessment of the Rhizospheric Microbiota of E. atrorubens

The quantity of mesophilic aerobic and facultative anaerobic microorganisms (QMAFAnM) in the rhizospheric soil of *E. atrorubens* from each studied site was determined by plating on Luria–Bertani (LB) agar medium. About 10 g of substrate was mixed with 90 mL of phosphate buffer (pH 6.5) and shaken in an orbital shaker at 180 rpm for 20 min at 28 °C. A series of dilutions of each sample was made and 100 µL was added to a Petri dish with LB agar nutrient medium supplemented with cycloheximide (75 mg L^{-1}) to suppress the growth of fungi. Inoculations on a nutrient medium were carried out with 2–5 dilutions, and 2 parallel inoculations were made from each dilution. For the growth of cultured bacteria, the plates were incubated for 3 days at 28 °C in a bacterial incubator (TSO-1/80 SPU, Smolensk, Russia). Colonies of bacteria were counted on the 3rd and 5th days of incubation, ignoring the dishes on which the number of colonies was less than 10 or more than 300. QMAFAnM was expressed in colony-forming units (CFU) per g of DW of soil.

The enzymatic activity of *E. atrorubens* rhizospheric soil was assessed by the activity of cellulose-degrading microorganisms (bacteria and fungi) [36].

To assess mycorrhizal colonization in the root system of *E. atrorubens*, root tips 1.0–1.5 cm were cross-sectioned to 20 µm with a freezing microtome MEP-01 (Technom, Ekaterinburg, Russia). Root sections (50 samples from each studied site) were analyzed using the light microscope Meiji MT 4300L (Meiji Techno, Saitama, Japan) at 100-x magnification [7]. The presence of pelotons or intracellular hyphal coils was determined within the root cortical cells. The percentage colonization was assessed as the proportion of sections containing pelotons compared to the total number of sections per plant.

2.5. Assessment of Plant Water Status

The transpiration rate was measured on the middle tier leaves (6–7 from the top) of *E. atrorubens* using an LI-6400XT portable infrared gas analyzer (LI-COR, Lincoln, NE, USA) [8].

Relative water content (RWC) and water saturation deficit (WSD) were measured using the floating disc method and calculated according to Hellmuth [37]. The fresh leaf cuttings (6 discs 0.9 cm^2 in diameter) were immediately weighed to obtain fresh weight (FW) and then saturated by submerging the sample in distilled water for 2 h. Afterwards, the surface water was blotted carefully and the discs were weighed to obtain the saturated weight (SW) and later dried for 24 h at 75 °C to determine the DW.

RWC was calculated using Equation (1):

$$\text{RWC (\%)} = (\text{FW} - \text{DW})/(\text{SW} - \text{DW}) \times 100 \quad (1)$$

WSD was calculated using Equation (2):

$$\text{WSD (\%)} = (\text{SW} - \text{FW})/(\text{SW} - \text{DW}) \times 100 \quad (2)$$

2.6. Assessment of Lipid Peroxidation and Non-Enzymatic Antioxidants

The lipid peroxidation was assessed by the content of oxidation products (malondialdehyde, MDA) according to Heath and Packer [38] in fresh leaves (0.3 g) homogenized with 4 mL of the reaction medium containing 0.25% thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) (*v*/*v*). Then the extract was boiled for 30 min, cooled on ice and centrifuged at 12,000× *g* for 15 min. The supernatant absorbance was measured at 532 and 600 nm. The TBA-reactive product concentration was calculated using the extinction coefficient (155 mM^{-1} cm^{-1}) and expressed in nmol MDA per g of DW.

The free proline and ascorbate content was determined as described previously with slight modifications [39]. The amount of proline was measured after leaf extraction (0.4 g) in 10 mL of boiling water (100 °C) for 10 min; then the reaction medium containing the prepared filtered extract and a mixture of ninhydrin reagent with glacial acetic acid (1:1:1; *v*/*v*) were placed in a boiling water bath for 30 min for staining and then cooled in ice rapidly. The proline content was quantified spectrophotometrically by PD-303 UV (Apel, Saitama, Japan) at 520 nm and calculated in mg per g of DW. The proline standard curve (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was made following the same protocol.

The ascorbate content was measured after the homogenization of leaves (0.3 g) in 3 mL of 2% (*w*/*v*) metaphosphoric acid. The homogenate was transferred into 15 mL Falcon tubes and the volume was made up with a mixture containing 2% metaphosphoric acid:0.21 M trisodium phosphate (3:2, *v*/*v*), pH 7.3–7.4. The extract was centrifuged for 3 min at 3000× *g* and the absorbance was measured at 265 nm against a blank containing the metaphosphoric acid–trisodium phosphate mixture. If necessary, the extract was further diluted. The ascorbate concentration was calculated in mg g^{-1} of DW using an extinction coefficient (1.655 × 10^4 M^{-1} cm^{-1}) and the molecular weight of ascorbate (176.1 g M^{-1}).

For the determination of the total content of phenolic compounds and flavonoids, the fresh leaves (0.3 g) were crushed and extracted with 10 mL of 80% ethanol for 24 h (in the dark). Then the resulting extract was filtered through the filter paper and used for the analysis. The total phenolic content was determined with the Folin–Ciocalteu reagent [40]. Briefly, 0.1 mL of the extract sample was reacted with 0.5 mL of 0.2 M Folin–Ciocalteu reagent for 5 min and then 0.4 mL 7.5% sodium carbonate solution (*w*/*v*) was added to the reaction mixture. The absorbance readings were measured with a multimode plate reader Infinite 200 PRO (Tecan, Grödig, Austria) at 760 nm after incubation at room temperature for 1 h. Gallic acid (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was used as a reference standard, and the results were expressed as mg gallic acid per g of DW.

The amount of flavonoids was determined using a modified method [41] after the reaction of the extract sample with an equivalent amount of 10% aluminum chloride ethanolic solution and then incubated at room temperature for 15 min, and the absorbance of the reaction mixture was measured at 415 nm. The amount of 10% aluminum chloride was substituted by the same amount of 80% ethanol in the blank. Similarly, the standard solution of rutin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was reacted with aluminum chloride to generate a calibration curve and the results were expressed as mg rutin per g of DW.

The extraction and determination of soluble protein and non-protein thiols were carried out as described by Borisova et al. [42]. The total content of soluble thiols was determined after reaction with Elman's reagent (5.5′-dithiobis (2-nitrobenzoic) acid) at 412 nm. The content of protein thiols was calculated by subtracting the amount of non-protein thiols previously obtained by precipitation of proteins with 50% trichloroacetic acid from the total soluble fraction. Reduced glutathione (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was used as a standard. The content of soluble protein was determined at 595 nm according to Bradford [43]. Bovine serum albumin was used as a standard.

2.7. Statistics

The study of the physicochemical characteristics of substrates, including analyses of macronutrients and heavy metals, was carried out on composite samples in quadruplicate. The granulometric analysis was performed in 4 independent replicates for each studied site. The physiological and biochemical analyses were performed in 6 replicates; data obtained over a two-year period were averaged. The tables and figures present the mean values (Means) and standard errors (SE).

After checking the normality using the Shapiro–Wilk's test and the homogeneity of variance using Levene's test, the differences between the studied orchid populations were determined with the non-parametric Kruskal–Wallis ANOVA by ranks and Mann–Whitney

U-test ($p < 0.05$). Different alphabetical letters in the tables and figures indicate a significant difference between the studied parameters.

3. Results

3.1. Physicochemical Characteristics of Substrates

The pH of the substrate on serpentine dumps (S2 and S3) was slightly alkaline, while the reference site (S1) was circumneutral (Table 2). The maximum value of ES was noted at Anatol'sky dump (S3); it was higher by 33% and 17% than in natural forest area (S1) and Shilovsky dump (S2), respectively. At the same time, the substrate moisture content was 2.3 times lower in S2 in comparison with S1 and S3 (Table 2).

Table 2. The pH values, electrical conductivity and nutrient content in substrates from studied sites.

Parameters	Sites		
	S1	**S2**	**S3**
pH	7.10 ± 0.01 b [1]	7.89 ± 0.02 a	7.65 ± 0.02 a
Electrical conductivity, μS cm^{-1}	166.80 ± 4.37 c	189.00 ± 0.58 b	222.00 ± 4.73 a
Substrate moisture content,% DW	19.50 ± 1.05 a	8.35 ± 0.47 b	19.20 ± 1.25 a
Available nutrients, mg kg^{-1} DW			
Nitrogen (N)	191.80 ± 27.04 a	32.20 ± 6.83 c	84.00 ± 17.81 b
Phosphorus (P_2O_5)	25.20 ± 8.80 b	31.70 ± 6.30 b	58.40 ± 11.70 a
Potassium (K)	136.18 ± 3.47 b	168.34 ± 2.46 a	127.24 ± 2.83 b

[1] Data is presented as Means ± SE ($n = 4$). Different alphabetical letters indicate a significant difference between the studied sites at $p < 0.05$.

The available nitrogen level in the soil from the natural forest community (S1) was considerably higher (by 3.3 times on average) compared to other sites (Table 2). At the same time, the differences in the content of phosphorus were not so significant. In the serpentine substrate of dumps, it was higher, especially in S3 (by 2.3 times). The maximum potassium concentration was observed in S2, while there were no differences between S1 and S3.

All the studied substrates were formed on serpentine rocks. However, in terms of particle size distribution, the following differences were revealed between them (Table 3).

Table 3. Granulometric composition of substrates from studied sites.

Site	Particle Size Distribution, %					
	>10 mm	**5–10 mm**	**2–5 mm**	**1–2 mm**	**0.25–1 mm**	**<0.25 mm**
S1	9.5 ± 0.3 c [1]	5.8 ± 0.2 c	3.8 ± 0.1 b	0.8 ± 0.2 c	14.6 ± 0.9 c	65.5 ± 2.4 a
S2	29.4 ± 1.1 a	16.6 ± 1.3 a	13.1 ± 0.9 a	9.3 ± 0.5 a	17.2 ± 1.2 b	14.4 ± 0.9 b
S3	26.3 ± 0.8 b	13.9 ± 1.2 b	12.6 ± 0.7 a	7.6 ± 0.3 b	22.7 ± 0.8 a	16.9 ± 1.3 b

[1] Data is presented as Means ± SE ($n = 4$). Different alphabetical letters indicate a significant difference between the studied sites at $p < 0.05$.

Substrates on Shilovsky (S2) and Anatol'sky (S3) dumps were very stony; fractions of large size (crushed stone and gravel) prevailed. Fractions <0.25 mm in size on S2 and S3 dumps accounted for only 15.7% on average, while in the natural forest community (S1), the proportion of small fractions was 4.2 times higher (Table 3).

3.2. Metal Content in Substrates and Plants

Data on the total content of metals in the studied substrates show that sites S1–S3 are distinguished by an increased content of many metals which is due to their serpentine nature. The total content of Mg in the substrate of serpentine dumps (S2 and S3) was on average 1.5 times higher than in the soil of the natural forest community (S1, Figure 3).

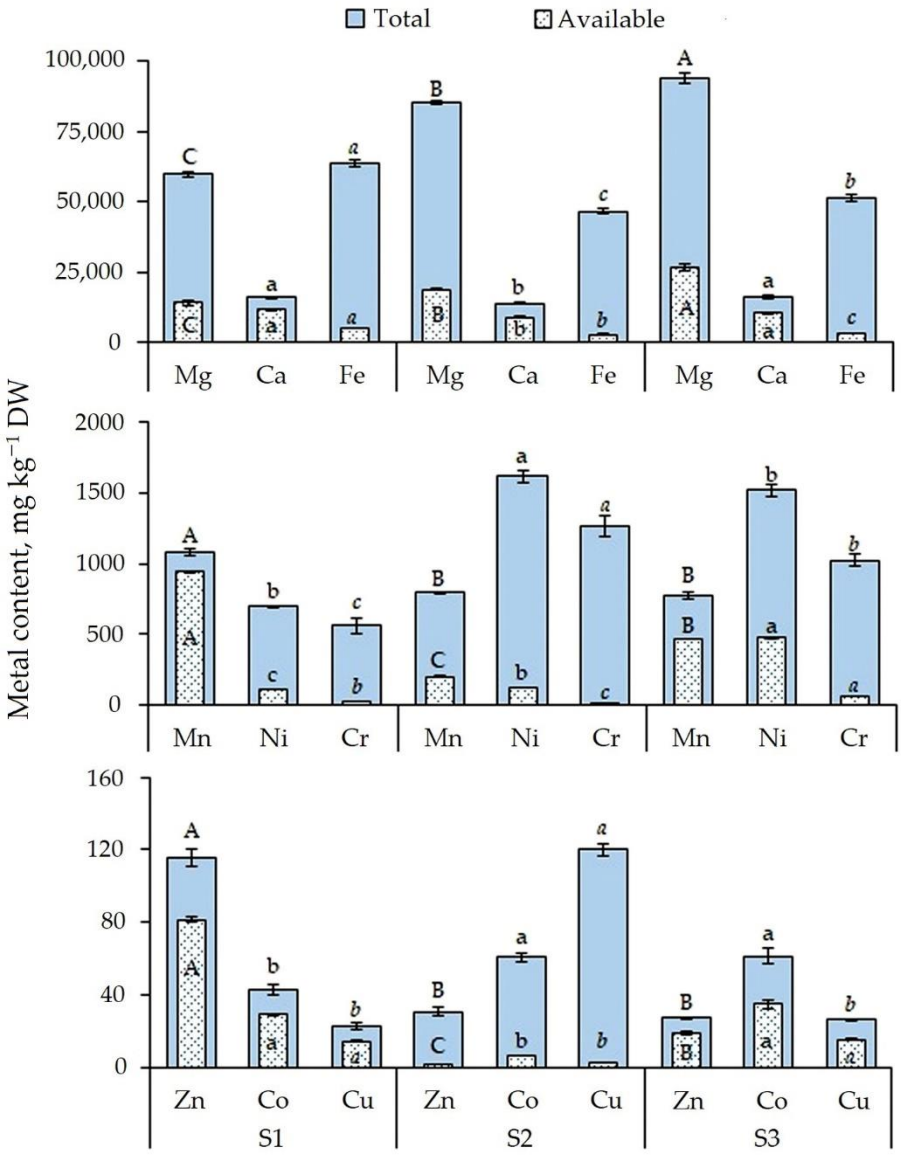

Figure 3. Total and available metal content in soil substrates from studied sites. Data is presented as Means ± SE (n = 4). Different alphabetical letters indicate a significant difference between the studied sites at $p < 0.05$.

The total content of Ni, Cr and Co in the dump substrate was also higher (by 2 times on average) than at the reference site (Figure 3). The reverse trend was noted for Fe, Mn and Zn; their maximum concentration was found in S1. In terms of the total Cu concentration, S2 was distinguished; the concentration being 5 times higher compared to other studied sites (Figure 3). The ratio of the total Mg:Ca on the dumps averaged 5.9, while in the natural community it was 3.7. The differences between the sites of the available Mg:Ca were not so considerable and on average it was 1.9.

The proportion of available metals in their total concentrations varied from 1.3% to 87% (Figure 3). The most significant part of the available form in all sites was found for Ca (75% on average). At two sites (S1 and S3), the proportion of available Mn, Zn, Co and Cu was more than 50% of the total concentration, while Fe and Cr in all sites were predominant in an inaccessible form. The Shilovsky dump (S2) was also distinguished by a low content of available Zn, Co and Cu (by 4.5% on average) (Figure 3).

The content of Fe, Mn, Ni, Zn and Cu in the rhizome + roots of *E. atrorubens* was higher on average by 2 times, while Cr and Co concentrations were higher by 4 and 7 times,

respectively, than in the leaves, excepting the macronutrients Ca and Mg: their average rhizome + roots to leaves ratio was close to 1 (Figure 4).

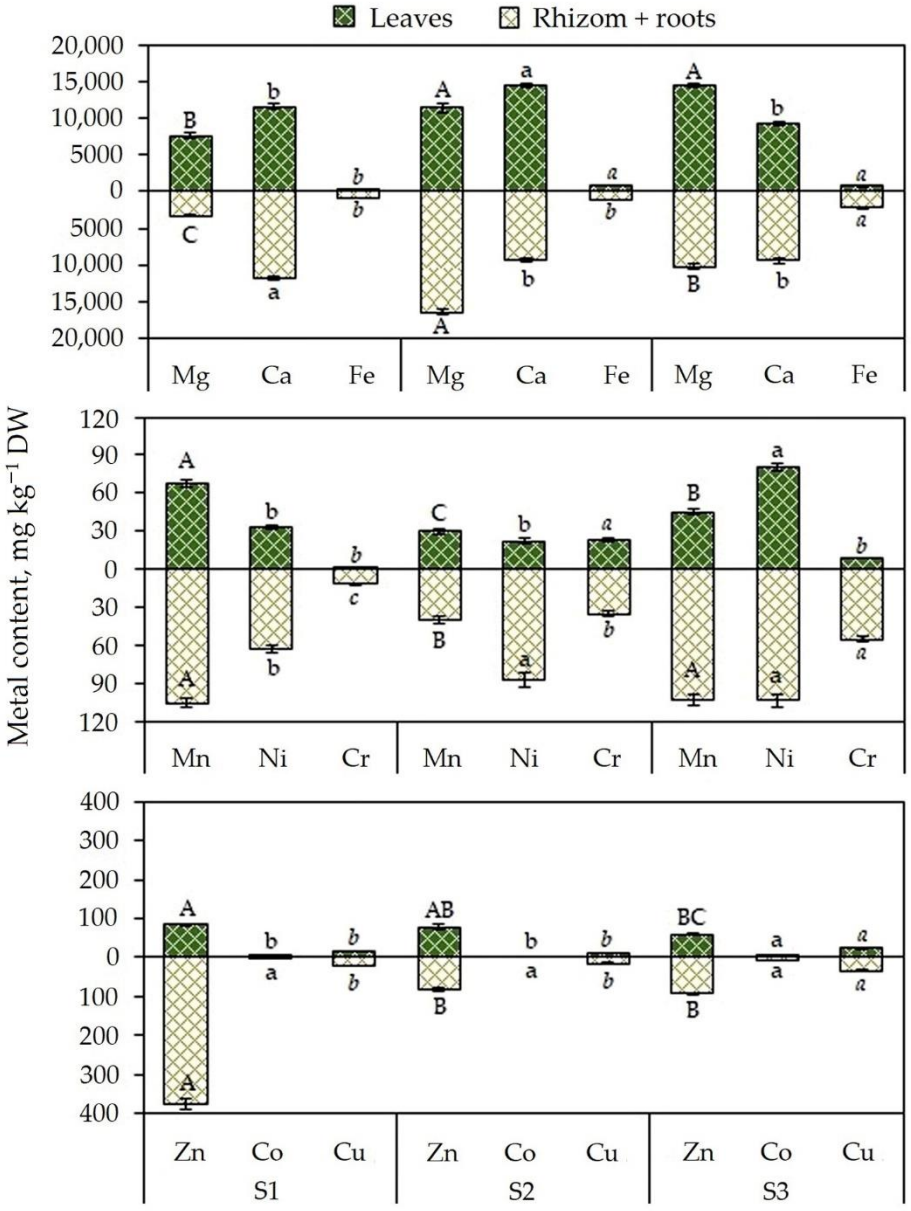

Figure 4. Metal content in the aboveground and underground organs of *E. atrorubens* growing on serpentine substrates. Data is presented as Means ± SE (n = 4). Different alphabetical letters indicate a significant difference between the studied sites at $p < 0.05$. kg^{-1} DW.

The content of Fe, Mg, Ni, Cr, Co and Cu in the leaves of *E. atrorubens* growing on dumps (S2 and S3) was on average 2 times higher than that in the natural community (S1). The opposite trend was noted for Mn and Zn (Figure 4).

3.3. The Rhizospheric Microbiota of E. atrorubens

The analysis of serpentine substrates collected in the root zone of *E. atrorubens* showed that the lowest density of bacterial cells (QMAFAnM) in the orchid rhizosphere was found on the Shilovsky dump (S2), while in the natural forest community (S1) and on Anatol'sky dump (S3), the number of rhizospheric bacteria was an order of magnitude higher (Table 4). The lowest enzymatic activity, estimated by the cellulose-decomposing microflora, was also noted in S2, which was 21 and 12 times higher than in S1 and S3, respectively (Table 4).

The degree of mycotrophy in *E. atrorubens* roots was the highest on the serpentine dumps (S2 and S3) in comparison with the natural forest community (Table 4).

Table 4. The characteristics of rhizospheric microbiota of *E. atrorubens* from studied sites.

Characteristics	Sites		
	S1	**S2**	**S3**
QMAFAnM, CFU g^{-1} DW	6.1×10^5	6.8×10^4	9.2×10^5
Enzymatic activity, %	6.5	0.3	3.6
Degree of mycotrophy, %	78	90	97

3.4. Plant Water Status

The intensity of transpiration in *E. atrorubens* leaves varied insignificantly compared to the reference site (S1). However, the highest values were noted in S2 plants (Shilovsky dump) which increased by 11 and 18% compared to S1 and S3 (Figure 5a). The RWC and WSD indexes entered the range of values of most plants and differed significantly only in S2 orchids (Figure 5b). In Shilovsky dump plants (S2) the water deficit increased by an average of 1.4 times compared to other orchid populations (S1 and S3).

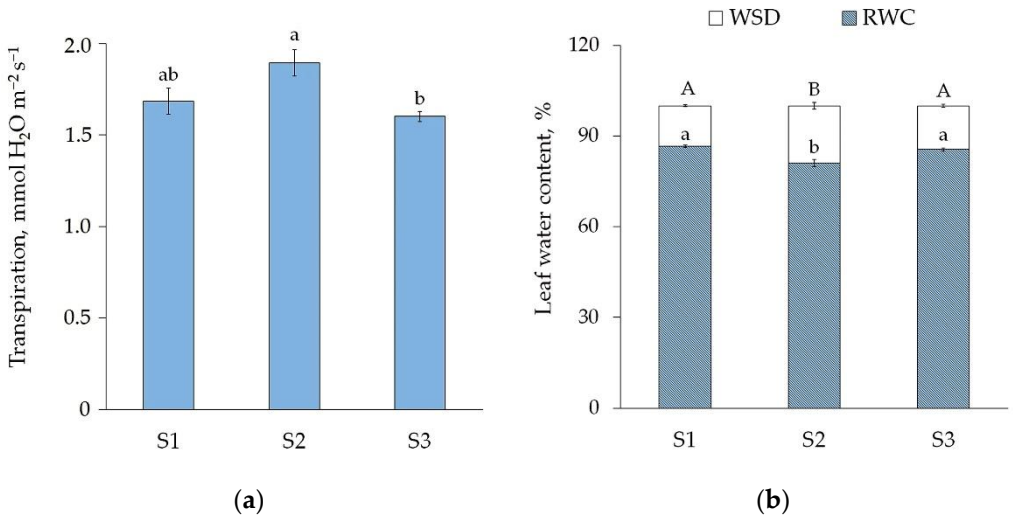

Figure 5. The water status parameters of *E. atrorubens* growing on serpentine substrates: (**a**) Transpiration rate; (**b**) Relative water content (RWC) and water saturation deficit (WSD). Data is presented as Means ± SE (n = 12). Different alphabetical letters indicate a significant difference between the studied populations at $p < 0.05$.

3.5. Redox Reactions of Plants

The study has shown that foliar MDA content in *E. atrorubens* growing on technogenic substrates was 1.5 and 1.2 times higher on the Shilovsky (S2) and Anatol'sky (S3) dumps, respectively, compared to S1 (Figure 6a).

The free proline content was 1.5 times higher in the leaves of S2 plants in comparison with S1 (Figure 6b). A slight increase (by 1.2 times) in the proline content was found in S3 plants (S3) compared to S1. The ascorbate amount in S2 plants was increased by 1.7 times in comparison with the reference site, however, there were no significant differences between S3 and S1 plants (Figure 6c).

The level of total soluble phenols in S2 orchids was higher by 1.5 and 1.6 times than in S3 and S1 plants, respectively (Figure 6d). In the leaves of plants growing on both dumps, the content of flavonoids was higher than in plants growing in natural conditions. The soluble protein thiols content was the same in all studied sites (Figure 6e). The content of non-protein thiols was higher by 1.8 times in plants growing on technogenic substrates compared to natural conditions.

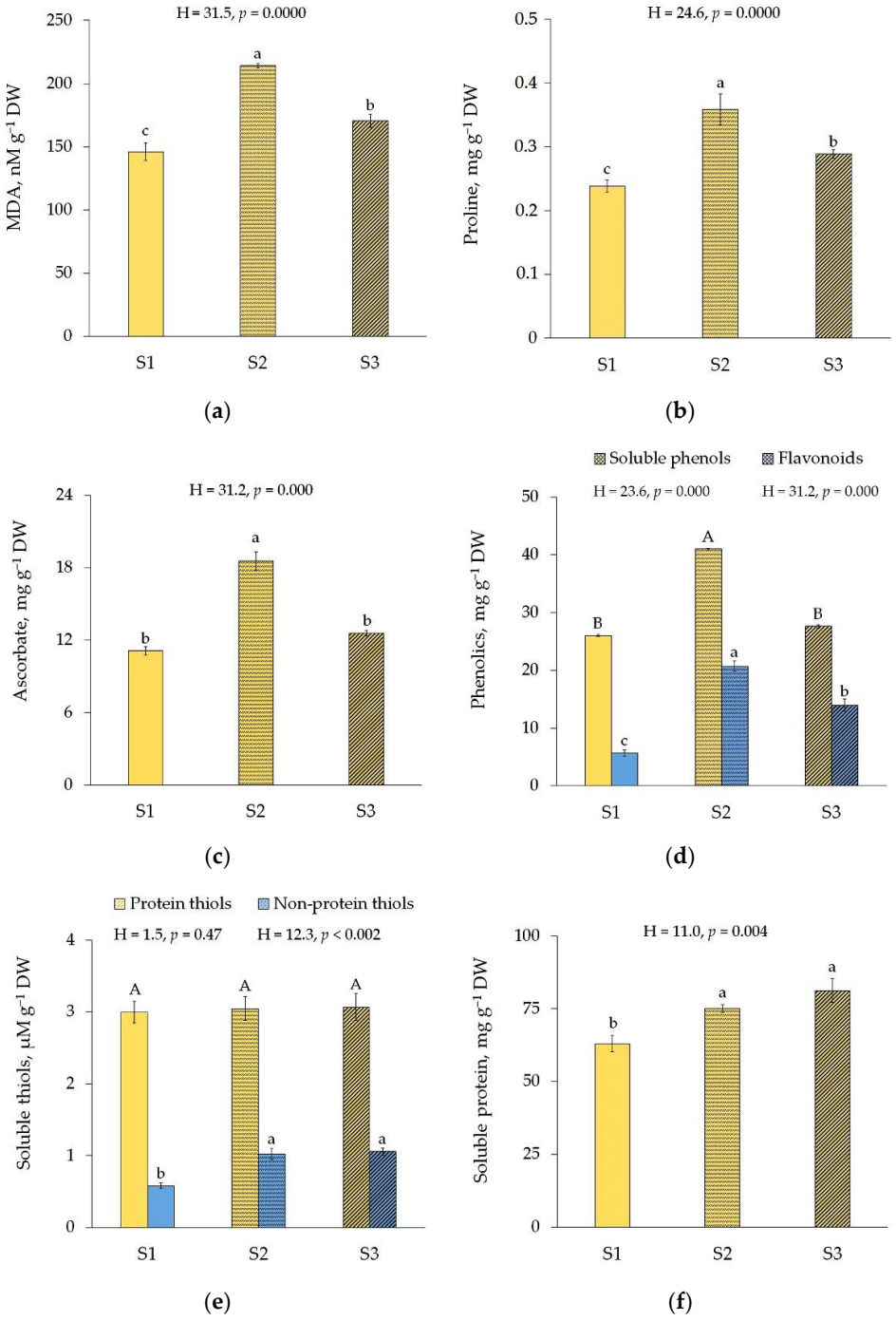

Figure 6. Pro- and antioxidant compounds in the leaves of *E. atrorubens* growing on serpentine substrates: (**a**) Malondialdehyde (MDA); (**b**) Free proline; (**c**) Ascorbate; (**d**) Soluble phenolics; (**e**) Soluble thiols; and (**f**) Soluble protein content. Data is presented as Means ± SE (n = 12). Different alphabetical letters indicate a significant difference between the studied populations at $p < 0.05$.

The content of soluble protein in *E. atrorubens* growing on technogenic substrates was increased by 1.3 times in comparison with the reference site (Figure 6f), however, there were no significant differences between S2 and S3 plants.

4. Discussion

The study of adaptive responses to abiotic stress in rare and endangered plants is an important prerequisite for their successful introduction [26]. The adaptive redox reactions

of orchid *E. atrorubens* were investigated on serpentine dumps post asbestos mining in comparison with a natural forest community. The serpentine substrates are unfavorable for plant growth due to their poor physicochemical properties: high stoniness; minimum amount of silty and clay particles; high content of iron, magnesium, nickel, chromium and cobalt, which are toxic to most plants and bacterial communities; and a low content of some macro- and micronutrients [16–18].

Although numerous studies have been carried out on plants in serpentine areas [6,7,9,19,44,45], there is little information concerning the adaptations of orchids abundantly growing on serpentine outcrops.

It was found that the substrates on the studied dumps (S2 and S3) were slightly alkaline and were characterized by low concentrations of available nitrogen and phosphorus, which is unfavorable for plant growth [46]. Moreover, the substrates on the dumps were characterized by high stoniness, which was associated with the mining of asbestos by the open method, after which overburdened rocks were mixed with loose rocks and enrichment wastes on the dumps [7].

The previous comparative study of *E. atrorubens* in two different forest plant communities of the Middle Urals (on serpentine and granite rocks) has shown that serpentine substrate differed by an extremely high concentration of total Mg which was 79-fold higher than in soil on granites. The high concentration of total Ni, Cr, Co and Fe was also found in serpentine substrate, where Ni, Cr, Co and Fe were 94, 59, 17 and 4 times higher than in granite ones, respectively [7]. The comparison of metal concentrations in the serpentine substrate on dumps with the averaged data on the serpentine rocks in the Urals is of particular interest. It was found that the total content of Mg, Fe, Ni and Cr in the serpentine dumps of the Anatol'sko-Shilovsky asbestos deposit was on average three times higher compared to the averaged data on serpentine substrates in the region reported by Teptina and Paukov [44]. However, a comparison of the obtained data with the information presented by Kierczak et al. [18] shows that the total and available nickel content was within the range of values for the serpentine soils of Lower Silesia (southwestern Poland). At the same time, the content of Cr and Co was slightly lower.

The previously conducted study showed that the possible reason for the increased *E. atrorubens* tolerance to high concentrations of metals in the serpentine soil was its ability to accumulate a higher amount of metal in its root system and prevent its transfer to the aboveground organs [7]. A similar trend was also found in this study. The content of all studied heavy metals (HMs) in *E. atrorubens* leaves was lower than in the roots. Nevertheless, the content of some metals (Ni and Cr) in the leaves of plants on serpentine dumps was higher than their critical concentrations reported by Kabata-Pendias and Mukherjee [47].

The high tolerance of *E. atrorubens*, as well as of some other species of the *Epipactis* genus, to elevated concentrations of HMs was evidenced by the reported results of other authors [4,9,29]. Many researchers attributed this to a well-developed mycorrhiza in the roots of orchids [15,29]. Orchid mycorrhiza, represented by pelotons, was found in the root cells of *E. atrorubens* from all studied sites. The percentage of mycorrhiza occurrence was higher in the dumps in comparison with the natural environment. This fact allows us to assume that mycorrhiza in this orchid plant make it more tolerant to the high concentrations of HMs in serpentine soils. It is also important to point out that the soil bacteria, along with micromycetes, are the most important components of the system of symbiotic relationships between orchid plants and microorganisms [48]. It is known that many endophytic and rhizospheric bacteria have the ability to stimulate plant growth through various mechanisms [31,48,49]. However, data on the ability of bacterial communities in the rhizosphere to promote the growth of orchids and increase their viability under abiotic stress conditions are fragmentary [30].

It should be noted that, despite the complex action of unfavorable edaphic factors on orchids colonizing serpentine dumps (S2 and S3), their local populations turned out to be more numerous than in the natural forest community (S1). Furthermore, it was found that *E. atrorubens* grows well on serpentine sites without showing any detrimental effects.

Obviously, this is partly due to the fact that in the natural ecotope the orchids experienced more pronounced phytocoenotic stress. The significance of serpentines as suitable habitats for the light-demanding orchids having low competitive ability was reported previously by Djordjević et al. [15].

On the whole, *E. atrorubens* demonstrated high tolerance to adverse abiotic factors. Therefore, it is important to identify the physiological and biochemical features that contribute to the colonization of this orchid on technogenic substrates. The study showed that *E. atrorubens* growing on serpentine dumps (S2 and S3) differed by the increased MDA content in the leaves compared to the ones in the natural forest community. An imbalance between the production of ROS and their neutralization often leads to oxidative stress in plants. At the same time, the orchid in transformed habitats demonstrated high tolerance to adverse abiotic factors. An important role in ensuring plant resistance to abiotic stress is played by the antioxidant system components, including non-enzymatic antioxidants [21,25]. It was found that lipid oxidative damage in orchid plants was accompanied by the synthesis of free proline, ascorbate, soluble phenolic compounds and soluble non-protein thiols in leaves. We suggested that *E. atrorubens* has adapted to the technogenic substrates by increasing the content of non-enzymatic antioxidants.

Proline is a proteinogenic heterocyclic amino acid which plays an important role in plant cells [27]. Osmotic adjustment is an important mechanism which alleviates some of the detrimental effects of water stress due to the accumulation of osmolytes [50]. The orchid plants from the Shilovsky dump (S2) had the highest content of free proline which was correlated with water deficiency ($r = 0.66$, $p < 0.05$). The increased water loss is possibly related to the characteristics of this technogenic habitat (strong substrate stoniness and intense insolation due to the lack of crown closure).

Water deficit stress tolerance is the result of the coordination of physiological and biochemical alterations at the organ, cellular and molecular levels [50]. Obviously, the increased accumulation of proline as the most important osmolyte contributed to the survival of *E. atrorubens* in unfavorable environmental conditions. It is known that proline is involved not only in osmoregulation, but also in the stabilization of proteins, membranes and subcellular structures. Proline is also able to chelate HMs, maintain cellular redox potential and participate in ROS neutralization [27,50].

The ascorbate represents a key molecule in plant metabolism. It plays a central role in several physiological processes such as photosynthesis, photo-protection, cell division, plant growth and stress responses [21,25]. Moreover, it performs a number of antioxidant functions and participates in the termination of chain reactions of oxidation organic compounds, preventing lipid peroxidation. In addition, ascorbate can directly react with ROS and participate in their neutralization, as well as restore other important antioxidants (phenolic compounds, tocopherol, etc.). [25,51]. The S2 *E. atrorubens* plants were characterized by an increased accumulation of not only free proline but also ascorbate content compared to other habitats, which indicates their active role in the adaptation to the abiotic stress.

Many phenolic compounds are known to have antioxidant properties. Phenolic substances readily interact with ROS. Initially, they are oxidized to phenoxyl radicals, the further oxidation of which leads to the formation of quinones. They can also chelate HMs and stabilize membranes, which limits the diffusion of free radicals and reduces the rate of lipid peroxidation [51,52]. As a rule, the synthesis of phenolic compounds is enhanced under stress. Previously, it was shown that the level of antioxidant activity in *Phalaenopsis* orchid hybrids was proportional to the concentration of phenolic compounds [53]. In *E. atrorubens* plants colonizing technogenic substrates (S2 and S3), the content of phenolic compounds in leaves directly correlated with the content of MDA ($r = 0.98$; $p < 0.05$). Particularly, many phenolic compounds, including flavonoids, were synthesized in S2 plants. It is important to point out that the content of flavonoids in S2 orchids was higher (by 4 times) than that in the hybrids of orchid *Phalaenopsis* reported by Minh et al. [53].

It should be noted that redox reactions, such as the increase in MDA content and the more active synthesis of free proline, ascorbate and phenolics, were manifested most clearly

in S2 plants (Shilovsky dump). Furthermore, the substrate on this dump was characterized by the lowest density of bacterial cells and lowest enzymatic activity compared to other sites. Perhaps this is due to the fact that S2 was represented by an emerging forest community without crown closure, while in S1 and S3 tree crown density was about 0.6.

The cell compounds containing SH-groups (thiols), which can be divided into protein and non-protein types, play an important role in the antioxidant protection of plants. Thiols can bind HMs and act as antioxidants, participating in the neutralization of ROS formed during oxidative stress [24,54]. An increased level of non-protein thiols was found in the plants growing on technogenic substrates (S2 and S3) compared to the natural conditions, which confirms their participation in protective reactions of plants to the action of HMs and other stressors. Additionally, many soluble proteins are involved in the antioxidant defense system of plants. They are capable of both directly chelating HMs and acting as enzymes, catalyzing the reactions of ROS neutralization [55].

Thus, the present study shows that an increased level of non-enzymatic antioxidant accumulation determines not only the ability of *E. atrorubens* to withstand negative environmental factors, but also to adapt to them successfully.

5. Conclusions

The overgrown dumps of the Anatol'sko-Shilovsky deposit today are areas with an infertile serpentine substrate containing large amounts of crushed stone and gravel, as well as higher concentrations of such heavy metals as Ni, Cr and Co, compared to natural habitat (2.0 times on average). Most of the studied metals predominantly accumulated in *E. atrorubens* rhizomes and roots compared to leaves (4 times on average). It was found that orchids colonizing serpentine dumps had more lipid peroxidation products (1.4 times on average), which demonstrated chronic oxidative stress. A comparative study of this orchid in the natural and transformed habitats allowed us to identify some compensatory adaptive reactions that contribute to its naturalization and distribution in technogenic substrates. The orchid plants demonstrated fairly high resistance to stressful conditions, probably due to the increased mycorrhization and more active synthesis of such non-enzymatic antioxidants as ascorbate, free proline, soluble phenolics including flavonoids and non-protein thiols. Further research will be aimed at studying the bacterial communities of the *E. atrorubens* rhizosphere and the estimation of the rhizobacteria characteristics that contribute to the growth and vital activity of the orchid under adverse environmental conditions.

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