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Adaptive Morphophysiological Features of *Neottia ovata* (Orchidaceae) Contributing to Its Natural Colonization on Fly Ash Deposits

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Abstract: In previous decades, some species of the Orchidaceae family have been found growing in man-made habitats. *Neottia ovata* is one of the most widespread orchids in Europe, however it is quite rare in Russia and is included in several regional Red Data Books. The purpose of this study was to compare the chemical composition and morphophysiological parameters of *N. ovata* from two forest communities of the Middle Urals, Russia: natural and transformed (fly ash dump of Verkhnetagil'skaya Thermal Power Station) for determining orchid adaptive features. The content of most of the studied metals in the underground parts (rhizome + roots) of *N. ovata* was considerably higher than in the leaves, which diminished the harmful effect of toxic metals on the aboveground organs. The adaptive changes in the leaf mesostructure of *N. ovata* such as an increase in epidermis thickness, the number of chloroplasts in the cell, and the internal assimilating surface were found for the first time. The orchids from the fly ash deposits were characterized by a higher content of chlorophyll *b* and carotenoids than plants from the natural forest community that evidenced the compensatory response on the decrease in chlorophyll *a*. The ability of *N. ovata* from the transformed habitat to maintain a relatively favorable water balance and stable assimilation indexes further contribute to its high viability. The study of orchid adaptive responses to unfavorable factors is necessary for their successful naturalization and introduction into a new environment.

Keywords: orchid; transformed ecosystems; fly ash; metals; adaptive responses; water exchange; leaf mesostructure; photosynthetic pigments; photosynthesis; plant introduction

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Highlights

Neottia ovata successfully colonize the fly ash dump (FAD) due to less phytocoenotic stress.

N. ovata plants from transformed habitat demonstrate high viability.

Sequestration of metals mainly in underground organs reduced harmful effect on orchid plants.

Natural orchid colonization of FAD was facilitated by adaptive structural and functional changes.

The FAD plants were characterized by the higher chlorophyll *b* and carotenoids content.

N. ovata from FAD maintained a relatively favorable water balance and stable assimilation indexes.

1. Introduction

The Orchidaceae family has a broad variety of more than 28,000 species distributed in about 763 genera and widespread from the Arctic tundra to tropical Brazilian rainforests [1,2]. It includes species with complex adaptations to pollination by specific insect

species and very different life strategies: from epiphytic to terrestrial, from evergreen to completely chlorophyll-free [3,4]. Studies have been conducted on orchids' taxonomy, morphology, ecology, breeding [5–7], pollination [8–10], genetics [11,12], mycorrhizal association [13–15], etc. At the same time, the physiological parameters of Orchidaceae species are still less studied and need much more attention [16].

Changing natural habitats have caused the extinction of many orchid species [17]. However, some orchids, especially in temperate regions of Europe and North America, have been found in anthropogenically disturbed territories, such as industrial dumps formed after the excavation and extraction of coal, iron, and some trace elements, and the fly ash dumps of thermal power plants [13,14,18–21].

The monitoring of vegetation restoration on disturbed lands in the Middle Urals, Russia, has shown that dumps from mining and processing industries are often colonized by some rare orchid species at the initial stages of the forest phytocoenoses formation [22–24]. Low competition in man-made habitats contributes to the conservation of the gene pool of Orchidaceae species. The local populations of *Neottia ovata* (L.) Bluff and Fingerh. (syn. *Listera ovata* (L.) R. Br. or Common twayblade) are of particular interest, as they have been found in recent years in disturbed territories of the Middle Urals, including fly ash dumps [22].

Common twayblade is one of the widespread orchids in Europe, especially in the British Isles [25]. However, this species is quite seldom encountered in Russia and has the status of a "rare species" in many regional Red Data Books, including the Red Book of Sverdlovsk Region [26]. This is a short-rhizome herbaceous perennial, mesophyte, European–West Asian, boreal-immoral species [3,25,27]. Like other orchids, *N. ovata* is characterized by low competitiveness. *N. ovata* grow on both fertile and poor soils. Sometimes it is found in disturbed habitats, along roadsides and railways, and in abandoned limestone quarries [4]. The *N. ovata* is a typical entomophile, the spectrum of pollinators is very wide [9]. This species reproduces well both by seeds and vegetatively [28]. For the germination of seeds in the first years of life, the presence of mycorrhiza is necessary [4].

Fly ash is considered a problematic form of solid waste throughout the world [29,30]. It is well known that fly ash substrates are characterized by low microbiological activity, an insufficient supply of nutrients, especially nitrogen, and adverse physicochemical properties [31]. Moreover, fly ash may also contain toxic concentrations of As, Cd, Cr, Pb, Co, Cu, etc. [29–31].

Different plant species growing in stressful environments show great variation in their tolerance mechanisms [29]. Unfavorable abiotic factors affect photosynthesis, respiration, water regime, and mineral nutrition, leading to impaired growth and development [21–24]. Photosynthesis is the main fundamental process that determines the productivity of plants [32,33]. The leaves are the primary photosynthetic organs, serving as key sites where the absorption of light and CO_2 assimilation take place. The internal organization of the leaf is influenced by environmental factors such as the chemical properties of soil substrates, light, temperature, and water availability. Leaf structure is known to be highly plastic in response to growing conditions, varying greatly in morphology, anatomy, and physiology [32–34]. The investigation of plant leaf traits and its responses to environmental change has increasingly gained more attention in recent decades [34–37]. Maintaining the functional activity of the photosynthetic apparatus in stressful conditions is one of the essential prerequisites that allow plants to colonize transformed territories.

The purpose of this study was a comparative analysis of the chemical composition and the structural and functional parameters of *N. ovata* from natural and transformed (fly ash dump) habitats. This will allow us to identify the adaptive morphophysiological characteristics of this species that contribute to its colonization on infertile technogenic substrates.

2. Materials and Methods

2.1. Study Area

The research was conducted in the Middle Urals, Russia (subzone of the southern taiga). The southern taiga subzone is characterized as moderately cold in terms of heat supply and over-humidified in terms of moisture availability. The average annual temperature is 1.9 °C, the annual precipitation is almost 570 mm, and the hydrothermal coefficient is about 1.5 [32]. The fly ash dump formed by brown coal ash is located on an area of 1.25 km^2. The fly ash deposits formed after mining (1968–1970) were left for colonization by natural forest [22,31,38]. The investigation was carried out in the vicinity of Verkhniy Tagil town (Sverdlovsk region) during the period of orchid blooming (mid-July 2018–2019). All samples were collected during the same period (from 15 to 18 July) under similar weather conditions (temperature during the daytime was 23 ± 3 °C and the relative humidity was about 60%). Two naturally growing orchid populations were studied: P-1 (57°20′13″ N 60°01′43″ E) from the natural forest community (NFC) near Belorechka village and P-2 (57°20′45″ N 59°56′46″ E) from the fly ash dump (FAD) near Verkhnetagil'skaya Thermal Power Station, VTTPS (Figures 1 and 2). The studied area of each site was about 400 m^2.

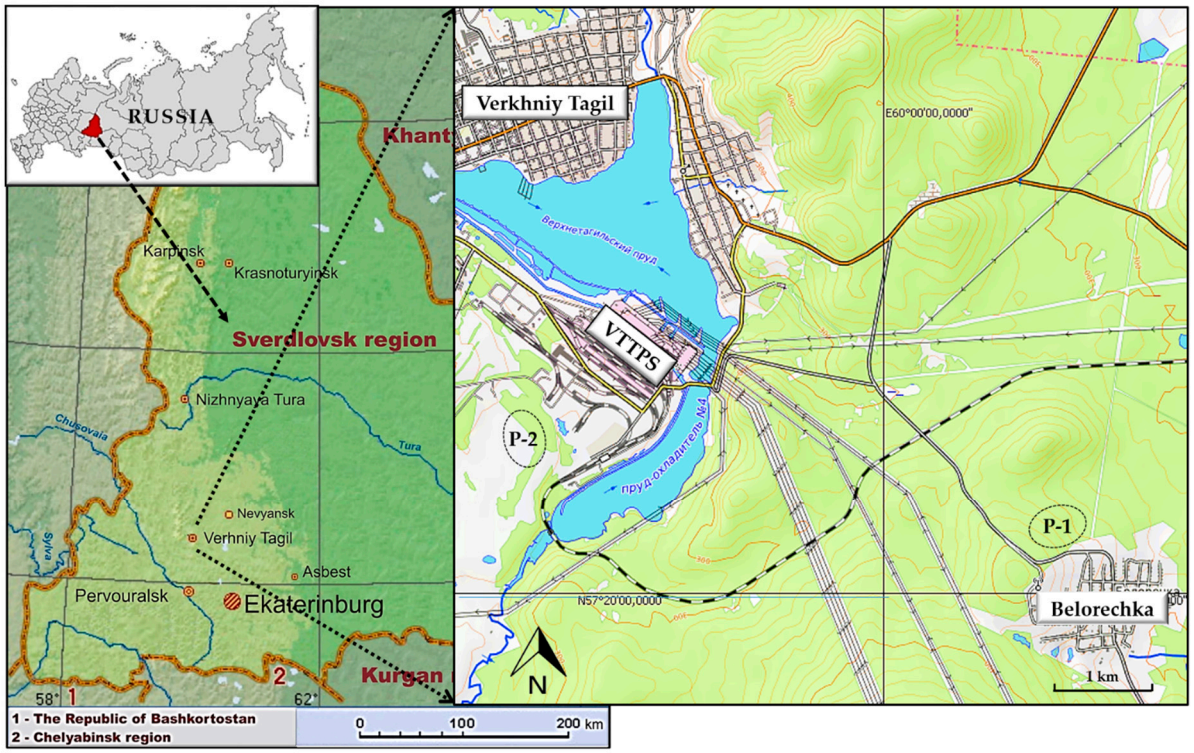

Figure 1. Map of Sverdlovsk region, Russia. Detailed presentation of the locations of studied *N. ovata* populations: P-1—from natural forest community near Belorechka village; P-2—from the fly ash dump near Verkhnetagil'skaya Thermal Power Station (VTTPS).

The natural forest community was represented by a mixed forest. The soil of this site was is sod-podzolic. The age of trees was between 80 and 100 years and the tree crown density was between 0.5 and 0.6. The height of the first layer of trees was 10–24 m and the second layer was 6–12 m. Coniferous species (*Picea obovata* Ledeb., *Larix sibirica* Ledeb., *Pinus sylvestris* L.) predominated in this forest. *N. ovata* individuals in P-1 grew mainly under the deciduous species *Betula pendula* Roth and *B. pubescens* Ehrh., as well as in small glades and meadows. The total projective shrub cover was 30–50% and contained *Tilia cordata* Mill., *Sorbus aucuparia* L., *Padus avium* Mill., *Rosa majalis* Herm., *R. acicularis* Lindl., and *Rubus idaeus* L. The total projective herbaceous cover was 70–80%, which reached up to 100% in the glades. Dominant among the herbaceous species were

Calamagrostis arundinacea (L.) Roth, *Aegopodium podagrária* L., *Círsium heterophyllum* (L.) Hill., *Brachypodium pinnatum* (L.) Beauv., *Geranium sylvaticum* L., *Lathyrus vernus* (L.) Bernh., *Anthoxanthum odoratum* L., *Alchemilla vulgaris* L., *Poa pratensis* L., *Melica nutans* L., *Vicia sepium* L., and *Ranunculus acris* L. The moss–lichen layer was weak. In total, more than 90 species grew on the investigated site and the species richness was 31 species per 100 m^2.

Figure 2. Flowering *N. ovata* plants from: (**a**) natural forest community (P-1) and (**b**) fly ash dump (P-2); (**c**) naturally colonized fly ash dump of VTTPS; *N. ovata* (**d**) inflorescence and (**e**) flower.

The orchid population P-2 was found in the young forest community formed during the natural revegetation of the fly ash deposits. Soil formation was proceeding according to zonal type in the fly ash substrate under the forest communities [38]. The tree crown density was 0.4, which reached up to 0.6 in some places. The 35–40-year-old forest community was dominated by *B. pendula*, *Populus tremula* L., *P. sylvestris*, *P. obovata*, and, less often, *B. pubescens*. In the undergrowth, there were singular instances of *P. obovata*, L. sibirica, and *Abies sibirica* Ledeb. The total projective shrub cover was 10–30% and contained *Salix myrsinifolia* Sm., *Sorbus aucuparia* L., *Padus avium* Mill., *Viburnum opulus* L., and *Chamaecytisus ruthenicus* (Fisch. ex Wołoszcz.) Klásková. The total projective herbaceous cover was 20–25%, in some places reaching 70%. The dominant species were *Platanthera bifolia* (L.) Rich., *Calamagrostis epigejos* (L.) Roth, *Amoria repens* (L.) C. Presl, *Pyrola rotundifolia* L., *Orthilia secunda* (L.) House, *Poa pratensis* L., *Festuca rubra* L., and *Equisetum arvense* L. The moss–lichen layer was weak. In total, more than 60 species grew on this site and the species richness was 19 species per 100 m^2.

2.2. Plant and Soil Substrate Sample Collection, Preparation and Analysis

From each site, no more than 20% of the total number of orchids in the studied populations were randomly selected to minimize the damage to these populations. Since the populations differed in terms of the number of individuals (39 and 194 in P-1 and P-2, respectively), four flowering orchid plants from natural forest population (P-1), and eight from the fly ash deposits (P-2), were carefully excavated with part of the soil substrate to preserve the underground organs (rhizome and roots). The studied plants from both the

sites were in the range of 50–60 cm in length. The samples were placed into separate sterile 10 L bags to minimize dehydration and transferred to the laboratory for further analysis.

The soil substrate samples (about 2 kg) were taken from each orchid root zone at a depth of 0–15 cm, and a composite sample was formed for analysis. Subsequently, the soil samples were air dried, homogenized, passed through a sieve (<2 mm), and preserved for physicochemical analysis (pH, electrical conductivity, total dissolved solids, total and available metal concentrations).

Before chemical analysis, the plants were carefully washed by ultrasonication (UM-4, Unitra Unima, Olsztyn, Poland) and finally with deionized water (Milli-Q system, Millipore SAS, Molsheim, France). The leaves, rhizome, and roots from each individual plant were separated and then dried for 24 h at 75 °C along with soil samples. Afterwards, the dried samples were weighed and digested with concentrated HNO_3 (analytical grade) using a MARS 5 Digestion Microwave System (CEM, Matthews, NC, USA). The available form of metals was analyzed by extracting the soil sample (5 g) with 10 mL of 0.5 M nitric acid solution as described earlier [24]. All the samples were prepared using deionized Millipore water. The concentrations of K, Ca, Mg, Fe, Zn, Mn, Pb, Cu, Ni, Cr, and Co in all the samples were determined using an atomic absorption spectrometer AA240FS (Varian Australia Pty Ltd., Mulgrave, Victoria, Australia) [24]. Standard reference materials (JSC Ural Chemical Reagents Plant, Verkhnyaya Pishma, Russia) were used for the preparation and calibration of each analytical batch. The calibration coefficients were maintained at a high level of no less than 0.99.

The bioconcentration factor (BCF) was calculated as the ratio of the metal concentration in the underground/aboveground organs to the available concentration in the soil. The translocation factor (TF) was calculated as the ratio of metal concentration in the leaves to the concentration in the rhizome + roots.

The pH, electrical conductivity (EC), and total dissolved solids (TDS) of the soil–water suspensions (1:2.5; *w/v*) were measured using a portable multivariable analyzer HI98129 Combo (Hanna Instruments GmbH, Graz, Austria). The total nitrogen and phosphorus content in the *N. ovata* leaves and rhizome + roots were measured spectrophotometrically at 440 and 640 nm, respectively, after wet digestion with an acid mixture of $HClO_4$ and H_2SO_4 (1:10; *v/v*). The total nitrogen was measured after the reaction with Nessler's reagent [39], whereas the total phosphorus was determined by standard method using ammonium molybdate in the acid medium [40].

2.3. Morphological, Anatomical Parameters and Mycorrhiza Assay

Twenty flowering plants from each population were used to study shoot and inflorescence length, the number of flowers, and the total leaf area under in situ conditions. To calculate the leaf area, each leaf was photographed on graph paper and digital image analysis was performed using special MesoPlant software (OOO SIAMS, Ekaterinburg, Russia). From the lower leaf of 10 plants about 30 leaf discs (0.7 cm diameter) were fixed in 3.5% glutaraldehyde solution in a phosphate buffer (pH 7.0, *v/v*) in order to study the mesostructural parameters: leaf mesophyll and epidermis thickness (μm), number of cells per unit leaf area (thousand cm^{-2}), chloroplasts per mesophyll cell (pieces), and cell and chloroplast volumes (μm^3). The transverse sections of the leaf discs were obtained using a freezing microtome MZ-2 (JSC Kharkov plant "Tochmedpribor", Kharkov, Ukraine). All measurements were carried out in 30 replicates using a Meiji MT 4300 L light microscope (Meiji Techno, San Jose, CA, USA). The quantitative parameters of the mesophyll were determined with a computer-assisted protocol based on MesoPlant software (OOO SIAMS, Ekaterinburg, Russia). The number of cells per unit of leaf surface area was counted in a Goryaev cytometer after tissue maceration in a 20% KOH solution (*v/v*) with heating at 80–90 °C. All other measurements were carried out on leaf discs preliminarily macerated with 5% chromic acid dissolved in 1 N HCl (*v/v*) [34].

The quantitative indices of the leaf mesophyll were determined according to Mokronosov [31], modified by Ivanova and P'yankov [34]. The cell volume per chloroplast (CVC,

μm^3) was calculated as the ratio of cell volume to the number of chloroplasts per cell. The chloroplast membrane index (CMI, cm^2 cm^{-2}) was calculated as the ratio of the total surface area of the outer membranes of chloroplasts to the unit of leaf surface area [35].

Fresh roots of *N. ovata* from both the studied sites were used for investigating mycorrhizal association. Root tips up to 1.5 cm were cut into 20 µm cross sections with a freezing microtome and analyzed under a Meiji MT 4300 L light microscope (Meiji Techno, San Jose, CA, USA) [24].

2.4. Physiological and Biochemical Parameters Assay

To measure photosynthesis and transpiration, freshly dug-up plants with bulk soil (as described earlier in Section 2.2) were transported to the laboratory and studied no later than 3 h after collection to minimize the dehydration.

The gas exchange (µM CO_2 m^{-2} s^{-1}) and transpiration rate (mM H_2O m^{-2} s^{-1}) were measured with the lower leaf of four plants using a LI-6400XT portable infrared gas analyzer (LI-COR, Lincoln, NE, USA) with a LED Light Source chamber (3 × 4 cm). The following parameters were set: operating at an ambient concentration of CO_2 and humidity, the temperature was +23 °C and the saturating light intensity of 1600 µM m^{-2} s^{-1}. This value of light intensity was experimentally established earlier by constructing average light curves. The CO_2 uptake was recalculated to mg of CO_2 per unit leaf area (dm^2), per mg of chlorophyll (Chl *a* + *b*), and per chloroplast (10^8) per hour; the transpiration rate was recalculated to g of H_2O per unit leaf area (dm^2) per hour. Subsequently, fresh leaf cuttings (0.7 cm diameter) from these leaves were used to measure the water exchange and photosynthetic pigment content.

The relative water content (RWC, %) and water saturation deficit (WSD, %) of the plant tissue were measured by floating disc method and calculated according to Hellmuth [41]. The fresh leaf cutting was immediately weighed to obtain FW and then saturated by submerging the sample in distilled water for 3 h. Afterwards, the surface water was blotted carefully, the discs were weighed to obtain the saturated weight, and later dried 24 h at 75 °C to determine the dry weight. The fresh weight (FW) to dry weight (DW) ratios were used for further calculations. Simultaneously, the part of the leaf discs was immediately used for photosynthetic pigment determination. Three discs from each plant (about 40–50 mg of FW) were homogenized in 2 mL of a cold 80% acetone solution (*v*/*v*) with addition of a small amount of $CaCO_3$ to protect the pigments from oxidation, and centrifuged at 8000× *g* for 10 min. The homogenate was decanted, acetone solution was added to the precipitate and stirred again; this procedure was repeated threefold until the precipitate was completely discolored. The content of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids (CAR) was determined spectrophotometrically ("APEL" PD-303 UV) at wavelengths of 470, 647, and 663 nm, respectively, and calculated according to Lichtenthaler [42], and expressed as mg g^{-1} DW. The physiological and biochemical parameters were determined in four biological and three analytical replicates.

2.5. Statistical Analysis

The values are presented as mean values of 5 replicates for the physicochemical analysis of the soil and the elemental analysis of plant samples, 30 replicates for structural characteristics, and 12 replicates for physiological and biochemical plant parameters with standard error (SE). After checking the normality by Shapiro–Wilk test and the homogeneity of variance by Levene's test, the differences between the studied orchid populations were determined with the nonparametric Mann–Whitney *U*-test, $p < 0.05$. The relationship between different parameters was determined by Spearman's rank correlation coefficient. Asterisks in the tables and figures indicate significant differences between the studied populations.

3. Results

3.1. Brief Description of Studied Populations

In NFC, *N. ovata* plants were found both as single individuals and in groups of up to three individuals. The total number of *N. ovata* plants in P-1 was 39, with a density of 0.10 individuals per 1 m^2; the age spectrum was dominated by flowering plants (71%).

The distribution of *N. ovata* plants in P-2 was uneven. The orchid population from FAD was young and vegetatively oriented, with pregenerative individuals predominating (78%). The total number of *N. ovata* plants in P-2 was 194 individuals and the density was 0.49 individuals per 1 m^2.

Orchid mycorrhiza, represented by pelotons, were found in the root cells of *N. ovata* from both studied populations. The intensity of mycorrhizal association was high and ranged from 96 to 98%.

3.2. Soil and Plant Composition

3.2.1. Physicochemical Characteristics and Metal Content in Soil Substrates

The pH of the soil substrates varied between acidic and slightly acidic for both sites. At the same time, the pH of the fly ash substrate was slightly higher than that of the natural forest soil (Table 1). The EC and TDS values in the soil substrate from NFC were higher (1.7-fold on average) than in FAD.

Table 1. The pH (water solution), electrical conductivity and total dissolved solids in soil substrates (0–15 cm depth) from the natural forest community (NFC) and the fly ash dump (FAD).

Site	pH (H_2O)	Electrical Conductivity (EC), µS cm^{-1}	Total Dissolved Solids (TDS), mg L^{-1}
NFC	5.80 ± 0.04 [1] (5.62–6.34) [2]	162.8 ± 11.3 (108.0–280.0)	89.9 ± 6.1 (51.0–145.0)
FAD	6.19 ± 0.03 * (5.79–6.32)	96.6 ± 7.2 * (46.0–146.0)	48.0 ± 3.7 * (23.0–73.0)

[1] Data is presented as mean ± SE (n = 5); [2] In the brackets are the minimum and maximum values. Asterisks (*) indicate significant differences between the studied habitats according to Mann–Whitney U-test ($p < 0.05$).

The total metal contents in the NFC soil were found in the following order: Fe > Ca > Mg > Mn > K > Zn > Pb > Cu > Cr > Ni > Co (Table 2). A similar trend was noted in the metal content distribution in the FAD substrate, with the exception of Mn and K that switched places. The largest difference between the two sites was found for Mn; its total and available content in the NFC soil exceeded its concentration in the FAD substrate by 3.3 times on average. The total contents of Ca, Mg, Zn, and Cu were also higher in the soil of the natural habitat than in the disturbed one, but the differences between the sites were less noticeable (1.3–2.0 times). Whereas, for K, Pb, and Cr, their content (both total and available) was higher in the FAD substrate (on average by 1.4 times). At the same time, there were no significant differences between the total Fe, Ni, and Co contents in both sites. The available concentration of most of the studied metals (namely, Ca, Mg, Fe, Zn, Mn, Cu, and Ni) in the NFC soil was higher compared to the FAD substrate (on average 2.0 times, Table 2). As for Co, there were no significant differences in the content between the sites studied.

Table 2. Total and available metal content in the soil substrates (0–15 cm depth) from the natural forest community (NFC) and the fly ash dump (FAD).

Metal	Total Content, mg kg^{-1} DW		Available Content, mg kg^{-1} DW	
	NFC	FAD	NFC	FAD
K	948.8 ± 87.1 [1] (735.8–1162.1) [2]	1256.2 ± 75.8 * (1080.8–1450.6)	211.6 ± 5.6 (198.0–225.4)	310.9 ± 3.8 * (290.6–326.3)
Ca	16,779.6 ± 856.1 (14,690.2–18,876.0)	8369.1 ± 437.2 * (7286.3–10676.0)	6644.8 ± 534.3 (5395.9–7823.9)	4439.9 ± 480.9 * (3098.7–6351.4)
Mg	5321.0 ± 329.9 (4515.1–6127.2)	2708.0 ± 602.6 * (999.8–4368.0)	1264.1 ± 47.2 (1149.0–1379.2)	792.4 ± 139.6 * (411.7–1243.8)
Fe	36,273.4 ± 1472.1 (32,670.0–39877.8)	33,087.7 ± 3008.1 (24,256.6–41777.9)	4810.1 ± 214.8 (4288.5–5332.0)	2592.9 ± 358.0 * (1601.5–3705.0)
Zn	269.9 ± 25.4 (208.8–332.6)	192.1 ± 10.2 * (159.0–224.1)	158.4 ±14.4 (123.3–193.3)	92.7 ± 4.7 * (77.8–111.6)
Mn	2043.4 ± 153.4 (1694.0–2393.2)	572.7 ± 114.6 * (270.8–957.0)	1388.2 ± 123.8 (1086.4–1690.2)	439.9 ± 93.0 * (166.6–712.5)
Pb	152.6 ± 7.8 (133.8–171.3)	180.0 ± 5.1 * (158.8–196.7)	69.8 ± 7.2 (58.2–81.3)	86.2 ± 3.0 * (74.3–98.2)
Cu	110.3 ± 5.4 (98.1–122.4)	69.2 ± 8.4 * (42.1–98.2)	83.1 ± 5.4 (70.2–96.5)	39.5 ± 3.7 * (26.8–51.9)
Ni	26.2 ± 4.1 (16.8–35.5)	22.9 ± 4.9 (9.1–37.5)	8.5 ± 0.6 (6.9–10.0)	4.5 ± 0.6 * (2.8–7.8)
Cr	41.7 ± 1.9 (37.6–45.9)	63.3 ± 3.1 * 49.5–72.8	4.8 ± 0.4 (4.1–5.6)	8.1 ± 0.5 * (5.9–10.1)
Co	5.4 ± 0.5 (3.9–7.2)	7.5 ± 0.9 (4.3–11.5)	3.4 ± 0.4 (2.3–4.3)	4.5 ± 0.4 (3.1–6.1)

[1] Data is presented as mean ± SE (n = 5); [2] In the brackets are the minimum and maximum values. Asterisks (*) indicate significant differences between studied habitats according to Mann–Whitney U-test ($p < 0.05$).

3.2.2. Macronutrient and Metal Content in *N. ovata*

The leaves of *N. ovata* from P-2 contained a smaller amount of total nitrogen, while the content of total phosphorus was higher than in the P-1 plants (by 1.2 times, Table 3).

Table 3. Macronutrient content in the aboveground and underground organs of *N. ovata* from the natural forest community (P-1) and the fly ash dump (P-2).

Macronutrient	Leaves, mg g^{-1} DW		Rhizome + Roots, mg g^{-1} DW	
	P-1	P-2	P-1	P-2
N	42.84 ± 1.30 [1]	23.08 ± 2.32 *	29.05 ± 3.92	28.15 ± 3.13
P	4.48 ± 0.04	5.48 ± 0.15 *	3.96 ± 0.03	2.92 ± 0.25 *
K	33.00 ± 0.61	46.97 ± 0.73 *	9.13 ± 0.36	12.70 ± 1.37 *
Ca	19.17 ± 0.28	18.13 ± 0.98	16.41 ± 0.62	15.57 ± 0.85
Mg	2.22 ± 0.08	1.54 ± 0.08 *	1.58 ± 0.09	1.32 ± 0.05

[1] Data is presented as mean ± SE (n = 5). Asterisks (*) indicate significant differences between the studied populations according to Mann–Whitney U-test ($p < 0.05$).

As for content of these nutrients in the *N. ovata* rhizome + roots, there were no significant differences in the nitrogen content between the studied orchid populations, although lower phosphorus content was noted in the plants growing on the fly ash substrate.

As expected, among the studied metals the *N. ovata* plants accumulated K, Ca, and Mg in the greatest amounts (Table 3). The potassium content in the both the aboveground and underground organs of *N. ovata* was 1.4 times higher than in the plants from the FAD while the studied populations did not significantly differ in terms of Ca and Mg content.

As for the other metals, they accumulated to the greatest extent in the *N. ovata* underground organs (Figure 3). The content of toxic elements such as Pb and Cr was higher in the rhizome + roots of the plants colonizing FAD (by 43 and 26%, respectively). In contrast, the content of essential Cu and Fe was lower (1.3 and 5.8 times, respectively). A similar tendency

was noted for Mn. The differences between the studied populations in terms of metal content in their leaves were less noticeable, with the exceptions of Pb and Fe (Figure 3).

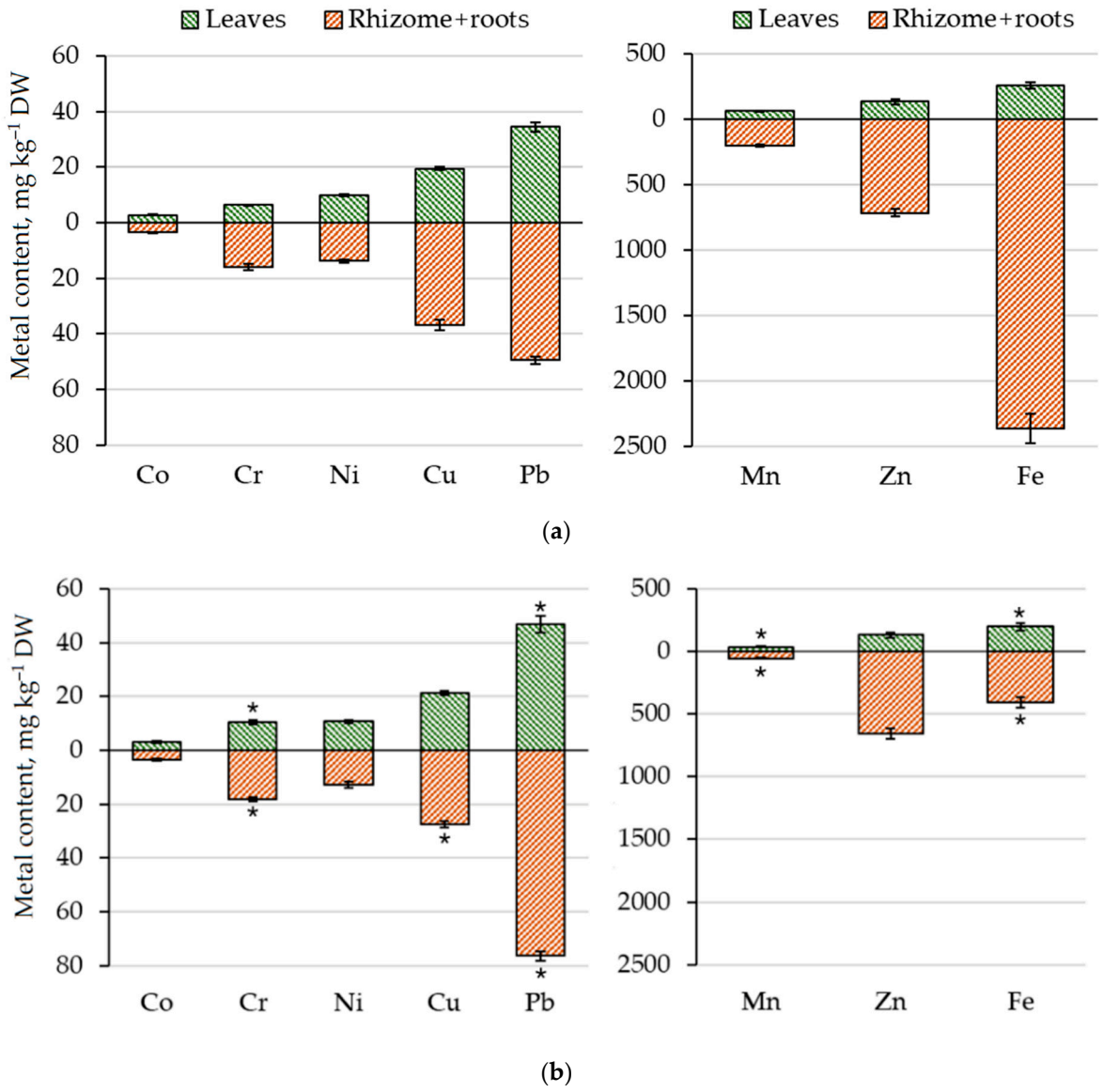

Figure 3. Heavy metal content in the aboveground and underground organs of *N. ovata* from: (**a**) the natural forest community (P-1) and (**b**) the fly ash dump (P-2). Data is presented as mean ± SE (n = 5). Asterisks (*) indicate significant differences between the studied populations according to Mann–Whitney U-test ($p < 0.05$).

The concentration of some trace metals (Zn, Cu, Cr and Co) in the underground organs correlated with their total content in the soil (on average r = 0.64; Supplementary Table S1). Moreover, for copper and chromium a significant correlation was also noted with regard to the available concentration in the soil (on average r = 0.66, Supplementary Table S2).

The BCF for macronutrients was found in the following order at both sites: K > Ca > Mg (Table 4). Potassium was released: its average BCF in the rhizome + roots of the orchid was 42, while in the leaves was 153. The plants from FAD had increased BCF values for zinc, which were several times higher than 1 and significantly higher than in NFC. The BCF for Ni and Cr were also greater than 1, while for other studied metals were ≤1.

Table 4. Bioconcentration factors (BCF) and translocation factor (TF) from underground to aboveground organs of *N. ovata* from natural forest community (P-1) and fly ash dump (P-2).

Metal	$BCF_{(Aboveground)}$		$BCF_{(Underground)}$		$TF_{(Aboveground/Underground)}$	
	P-1	P-2	P-1	P-2	P-1	P-2
K	155.94	151.09	43.13	40.86	3.62	3.70
Ca	2.89	4.08	2.47	3.51	1.17	1.16
Mg	1.75	1.95	1.25	1.67	1.41	1.17
Fe	0.05	0.08	0.49	0.16	0.11	0.48
Zn	0.85	1.39	4.50	7.11	0.19	0.20
Mn	0.04	0.08	0.15	0.13	0.31	0.62
Pb	0.49	0.54	0.71	0.82	0.70	0.66
Cu	0.23	0.54	0.44	0.70	0.53	0.77
Ni	1.17	2.40	1.62	2.87	0.72	0.84
Cr	1.34	1.30	2.99	2.24	0.45	0.58
Co	0.84	0.82	1.04	0.94	0.81	0.88

3.3. Morphological and Anatomical Characteristics of N. ovata

The orchid plants growing on the fly ash substrate (P-2) had a lower shoot and inflorescence length, and number of flowers (by 30, 27, and 20%, respectively) compared to P-1, but at the same time they had a 1.4-fold larger leaf area (Table 5).

Table 5. Morphological characteristics of the flowering individuals of the *N. ovata* populations from the natural forest community (P-1) and the fly ash dump (P-2).

Parameters	Populations	
	P-1	P-2
Shoot length, cm	60.2 ± 7.2 [1] (46.0–69.5) [2]	45.0 ± 2.1 * (23.0–69.0)
Inflorescence length, cm	18.8 ± 4.9 (13.0–28.5)	14.8 ± 1.1 (7.2–30.0)
Number of flowers, pcs.	29.0 ± 8.3 (17.0–45.0)	23.7 ± 2.1 (7.0–46.0)
Upper leaf area, cm^2	38.9 ± 8.7 (12.9–60.6)	48.1 ± 5.8 (22.8–87.5)
Lower leaf area, cm^2	33.7 ± 11.9 (15.1–63.6)	53.6 ± 7.2 (24.1–101.4)

[1] Data is presented as mean ± SE (n = 20); [2] In the brackets are the minimum and maximum values. Asterisks (*) indicate significant differences between the studied populations according to Mann–Whitney U-test ($p < 0.05$).

The *N. ovata* leaves have a homogeneous mesophyll structure. The study showed that the orchids colonizing the fly ash substrate were distinguished by a thicker epidermis (by 14%) and lower mesophyll thickness (by 6%), compared to individuals from the natural habitat (Figure 4a,b).

There were no significant differences between the studied populations in terms of the number of mesophyll cells (Figure 4c), and their surface area and volume (Supplementary Table S5), whereas an increased number of chloroplasts both per cell (by 18%) and per unit cell area (by 13%), was noted for P-2 plants, compared to their P-1 counterparts (Figure 4d; Supplementary Table S5). The cell volume per chloroplast in the leaves of *N. ovata* from FAD was lower (Figure 4e) than in plants from NFC, while the reverse trend was observed for the chloroplast membrane index (Figure 4f).

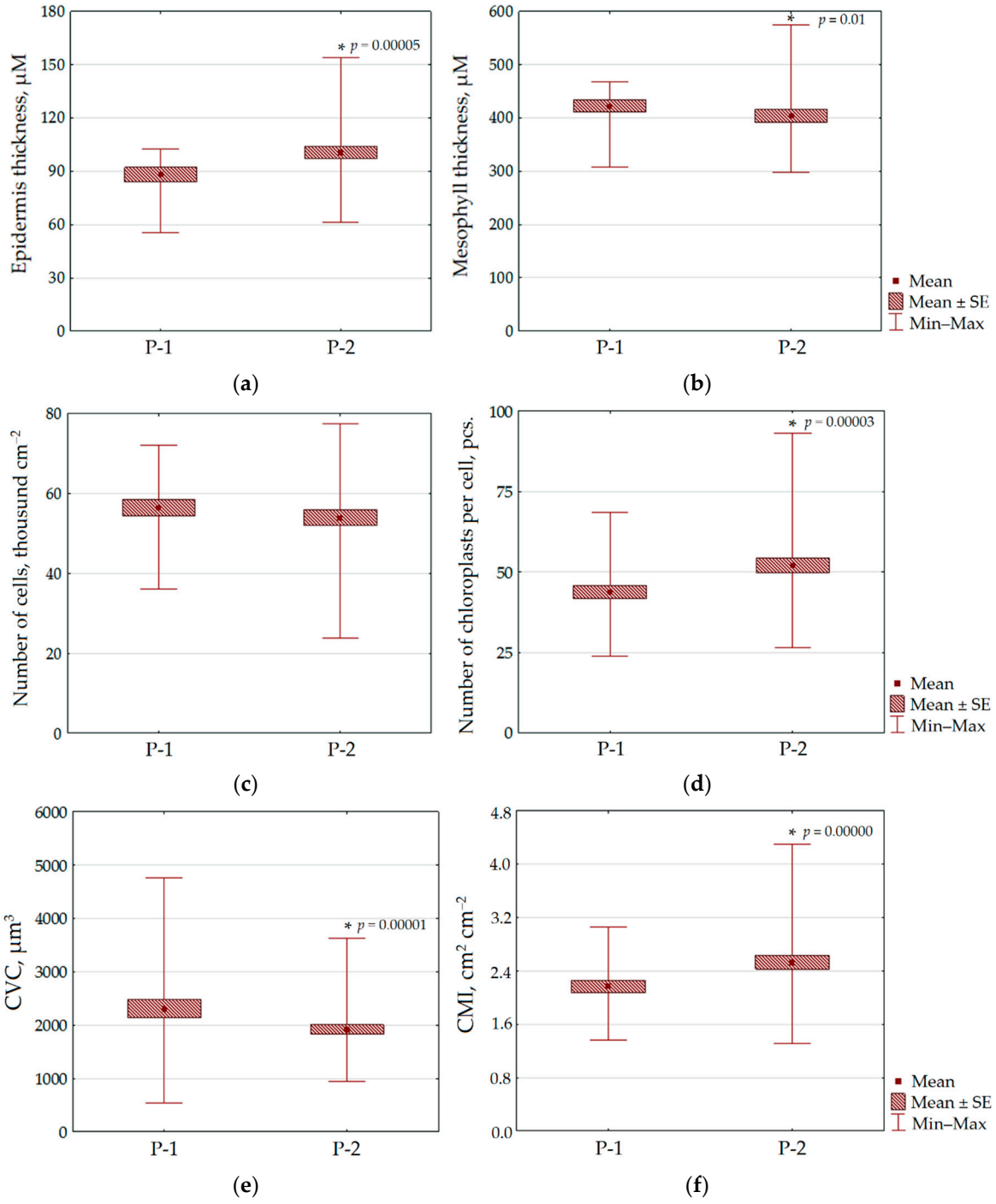

Figure 4. The mesostructure parameters of the lower leaf of *N. ovata* from the natural forest community (P-1) and the fly ash dump (P-2): (**a**) epidermis thickness; (**b**) mesophyll thickness; (**c**) number of cells; (**d**) number of chloroplasts per cell; (**e**) cell volume per chloroplast (CVC); (**f**) chloroplast membrane index (CMI). The small solid square indicates mean values (n = 30); boxes present mean ± SE; the whiskers are the minimum and maximum values. Asterisks (*) indicate significant differences between the studied populations according to Mann–Whitney U-test ($p < 0.05$).

3.4. Physiological and Biochemical Parameters of N. ovata

As shown in Figure 5a, the leaves of *N. ovata* from the natural habitat contained 2.5 times higher Chl *a* than Chl *b*. The Chl *a* content in the P-2 plants was 1.6 times lower than in the P-1 plants. In contrast, the Chl *b* and carotenoid content in the orchids on the fly ash substrates was higher (14% and 33%, respectively). A 1.9-fold decrease in Chl *a*/*b* and

Chl (*a* + *b*)/CAR ratios was observed in plants from FAD while the (CAR+ Chl *b*)/Chl *a* ratio increased almost 2 times compared to plants from the NPC site.

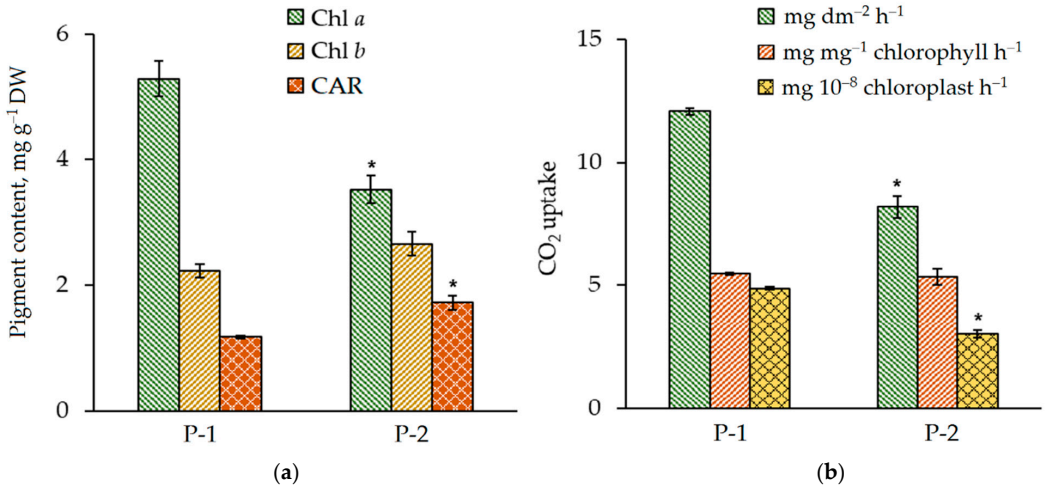

Figure 5. The photosynthetic parameters of the leaves of *N. ovata* from the natural forest community (P-1) and the fly ash dump (P-2): (**a**) photosynthetic pigment content; (**b**) intensity of CO_2 assimilation. Data is presented as mean ± SE (n = 12). Asterisks (*) indicate significant differences between the studied populations according to Mann–Whitney U-test ($p < 0.05$).

In addition, a high positive correlation between Chl *a* and available content of Cu, Ni, Fe, Mn, Mg, and Ca in soil (on average, r = 0.86, Supplementary Table S3) and negative correlation between Chl *a* and total Pb and Cr content in the leaves (on average, r = −0.86; Supplementary Table S4) were found for *N. ovata* plants.

The data on CO_2 assimilation intensity (Figure 5b) showed that its uptake per unit area and per chloroplast per hour in P-2 plants was 1.7 times lower than in P-1 plants. At the same time, there were no significant differences between the populations in terms of the CO_2 uptake per mg of chlorophyll per hour (Figure 5b).

The *N. ovata* plants on the fly ash substrate had a lower intensity of transpiration compared to the plants from the natural habitat (by 1.4 times, Figure 6a). The relative water content and water saturation deficit indexes entered the range of values of most plants and did not differ much between sites (Figure 6b). However, P-1 plants experienced a greater lack of moisture than P-2.

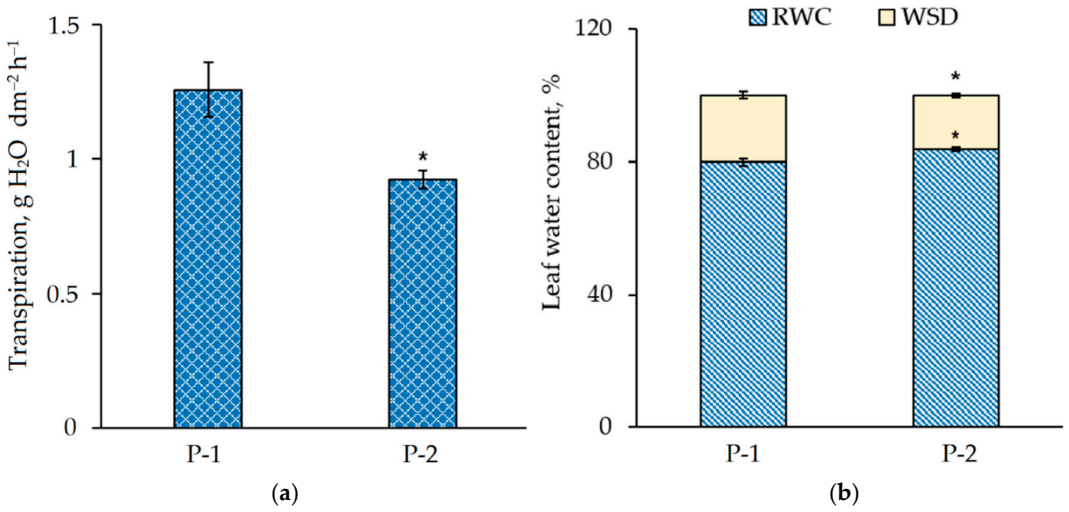

Figure 6. The water exchange parameters in the leaves of *N. ovata* from the natural forest community (P-1) and the fly ash dump (P-2): (**a**) transpiration intensity; (**b**) relative water content (RWC) and water saturation deficit (WSD). Data is presented as mean ± SE (n = 12). Asterisks (*) indicate significant differences between the studied populations according to Mann–Whitney U-test ($p < 0.05$).

4. Discussion

The present study aims to identify the adaptive responses of the rare orchid *N. ovata* that contribute to natural colonization under the adverse conditions of a fly ash deposits. No similar investigations have been carried out in the Middle Urals. A comparative analysis of the structural and functional characteristics of *N. ovata* plants in disturbed (fly ash dump of VTTPS) and natural forest ecosystems are vital for achieving this goal.

It is well known that fly ash substrates are characterized by unfavorable physicochemical properties, which depend on the type and origin of the coal, the conditions of combustion, the type of emission control devices, and the storage and handling methods [29–31,43]. The pH values of fly ash can vary from 4.5 to 12, depending on the coal type [29]. As a rule, fly ash formed during the combustion of brown coal is alkaline [29]. The lowered pH value of the FAD substrate is obviously explained by the fact that soil formation in this area had proceeded according to the zonal type (under the conditions of a flushing water regime) [38]. According to Gajic' et al. [29], unweathered fly ash had high values of electrical conductivity (150–352 μS cm^{-1}), which indicate a large amount of soluble salts, while EC values usually decrease (101–217 μS cm^{-1}) in weathered fly ash. In general, plants growing during the weathering of fly ash improve the physicochemical properties of the fly ash substrate. It was found that EC of the FAD substrate was reliably lower compared to the NFC substrate. This is associated with significantly lower available concentrations of most of the studied metals in the FAD substrate, which is also confirmed by the low TDS values.

As noted, toxic concentrations of Cr, Pb, Cd, As and other metal(loid)s are often a limiting factor that reduce the rate of natural colonization of fly ash dumps [30]. The studied metal content in the soil of both sites did not exceed the maximum permissible concentrations [44]. All the studied metals, with the exception of macronutrients (Ca, Mg, and K), accumulated to the greatest degree in the roots of *N. ovata*. The increased level of trace metal accumulation in *N. ovata* roots indicates the functioning of barrier mechanisms and contributes to the implementation of an ontogenetic program [34]. Nevertheless, increased Co, Cr, Ni, and Pb content in the leaves was noted (above the normal level), which corresponds to an excessive or toxic concentration [44].

The plants growing on FAD showed the greatest difference in terms of potassium in both the aboveground and underground organs. This is due to the higher concentration of potassium in the fly ash substrate. Potassium plays a vital role in such important processes as photosynthesis, growth, assimilate transport, water exchange, etc. [45]. Thus, the high ability of *N. ovata* to absorb potassium and translocate it to leaves is one of its adaptive responses.

Fly ash substrates are known to be very poor in nitrogen content [29,30,38]. Nevertheless, its content in the orchid plants from FAD was within normal limits (at the level of average values) [45]. These results confirm the existing view that orchids can effectively assimilate nitrogen even from soils poor in this element [46]. In contrast, the total phosphorus content was higher in the leaves of *N. ovata* from FAD compared with those from NFC. Since phosphorus is involved in many biochemical, energy, and physiological processes [45], its accumulation in leaves can obviously be regarded as an adaptive response.

The *N. ovata* individuals from FAD were characterized by lower values of shoot height, inflorescence length, and the number of generative organs. These were compensated by an increase in the area of the assimilative organs.

Most orchids, especially species with thin leaves, assimilate carbon dioxide through a C_3-pathway [16]. The intensity of photosynthesis depends on the activity of photosynthetic enzymes and pigment concentration [32]. On the other hand, this is largely related to the leaf blade's anatomical and morphological characteristics, which determine the optical properties and diffusion rate of CO_2 to the carboxylation centers [33]. Under the stress factors, changes in the mesostructure of the photosynthetic apparatus can take place as an adaptive reaction [34,35].

The studied *N. ovata* populations revealed a lack of significant differences in leaf blade thickness. At the same time, the properties of the substrate affected the thickness of the mesophyll and epidermis: a thinner mesophyll and a thicker epidermis were characteristic of plants from FAD. This is a protective response which is probably associated with increased atmospheric dust at dump sites [29].

An increased number of chloroplasts per cell in the *N. ovata* growing in a transformed habitat can be regarded as a compensatory adaptive reaction to the lack of chlorophylls, the content of which was significantly lower in plants from the fly ash deposits. The cell volume per chloroplast is a parameter that indicates the size of the cell volume, which is provided by metabolites as well as energy substrates due to the activity of one chloroplast [34,35]. A significant decrease in this indicator in *N. ovata* from FAD, compared with NFC, is explained by an increase in the number of chloroplasts in its mesophyll cells, while the cell volume remained practically unchanged.

The chloroplast membrane index is an integral indicator of the photosynthetic apparatus [34]. Obviously, the more significant CMI observed for P-2 plants is explained by the significant increase in the number of plastids in mesophyll cells. Thus, the greater development of the leaf's internal assimilation surface in orchid plants growing on the fly ash substrate is apparently associated with lower values of tree crown density and the total projective cover of the grass-shrub layer and as a consequence, greater lighting.

It is well reported that the photosynthetic apparatus of plants ensures their vital activity in various environmental conditions [47]. The pigment complex of plants is highly sensitive to the effects of adverse factors and capable of adaptive changes. Therefore, the analysis of the content and ratio of photosynthetic pigments is of great importance in assessing the resistance of plants to various stress factors [48]. Chlorophyll *a* in *N. ovata* leaves proved to be the most sensitive to the adverse conditions in the fly ash substrate, showing significant reductions. This can be explained by a lower nitrogen content since it is one of the most important components of green pigments [49] and a high concentration of some toxic metals (perhaps, Cr and Pb). It is well known that an excess of toxic metals in plant cells can cause structural changes in chloroplasts, inhibit key enzymes in chlorophyll synthesis, and cause the destruction of pigment molecules [24,29,35].

The degree of photosynthetic apparatus activity and its resistance to unfavorable external stressors are often evaluated by the photosynthetic pigment ratio. A comparison of pigment ratios showed the decrease in the Chl *a/b* and Chl $(a + b)$/CAR ratios in plants from FAD. This fact is explained by a significantly lower Chl *a* concentration, while Chl *b* and carotenoid content were increased. The ratio of the sum of the auxiliary pigments (Chl *b* + CAR) to Chl *a*, characterizing the share of antenna forms, significantly increased in *N. ovata* plants growing in FAD. Obviously, the activation of the synthesis of auxiliary pigments is a compensatory reaction that contributes to better absorption of light for photosynthesis. However, carotenoids in chloroplasts perform not only antenna and photoprotective functions, but also an antioxidant one. Carotenoid molecules can interact with reactive oxygen species (ROS) due to double bonds [50]. Thus, an increase in the carotenoid content in plants growing on fly ash substrate is most likely a response to stress and is aimed at combating ROS.

The absence of significant changes in the assimilation index (mg CO_2 mg^{-1} chlorophyll h^{-1}) indicates that the unfavorable conditions of the fly ash substrate did not cause significant damage to the chlorophyll molecules, since its functional ability was preserved [49].

The absorption of CO_2 and the transpiration of water by plant leaves occurs through the stomata. Accordingly, the rate of CO_2 assimilation and transpiration per unit area showed similar changes. The level of moisture deficiency in plants can be associated with the intensity of water evaporation. The results obtained are consistent with the data of measuring transpiration for each plot. Therefore, transpiration was more intense in orchids from NFC, than from FAD, which caused a higher water deficit. In addition, a greater moisture deficit in plants from a natural habitat can be explained by the high value of a

projective vegetation cover. Consequently, there was probably competition between the plants for resources, including water.

5. Conclusions

The present study revealed those adaptive structural and functional features of *Neottia ovata* that contribute to its survival strategies in a transformed habitat. Despite the adverse edaphic conditions of the fly ash dump, the population size of this species was noticeably higher than that in natural forest community. This is due to less phytocoenotic stress on the fly ash deposits while the population of *N. ovata* in its natural habitat experiences higher level of competition. Moreover, the natural colonization of *N. ovata* on the fly ash substrate was facilitated by adaptive changes in the mesostructure parameters of the leaves, such as an increase in epidermis thickness, the number of chloroplasts in the cell, and the internal assimilating surface. The *N. ovata* plants colonizing the fly ash dump were characterized by the higher chlorophyll *b* and carotenoids content compared to plants growing in the natural forest community that evidenced the compensatory response on the decrease in chlorophyll *a*. Furthermore, *N. ovata* growing on the fly ash substrate retained a relatively favorable water balance, which also contributed to its high resistance to adverse environmental conditions.

The content of most of the studied metals in the underground parts (rhizome + roots) of *N. ovata* was considerably higher than in the leaves, which diminished the harmful effect of toxic metals on the aboveground organs, including the generative ones. Despite the lower content of most of the macro- and micronutrients in the fly ash substrate and the higher concentration of some toxic heavy metals (lead and chromium), the plants from the transformed ecosystem showed high viability.

The study of the morphophysiological features of orchids in technologically disturbed habitats is necessary for developing measures to protect the gene pool of rare plant species and to solve applied problems associated with identifying optimal conditions for naturalization and introduction into a new environment.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7050109/s1, Table S1: Spearman's correlation between total metal content in the soil with metals in leaves and rhizome + roots of *N. ovata* from the natural forest community and the fly ash dump; Table S2: Spearman's correlation between available metal content in the soil with metals in leaves and rhizome + roots of *N. ovata* from the natural forest community and the fly ash dump; Table S3: Spearman's correlation between total and available metal content in the soil with physiological parameters of *N. ovata* from the natural forest community and the fly ash dump; Table S4: Spearman's correlation between metal content in the leaves and rhizome + roots with physiological parameters of *N. ovata* from the natural forest community and the fly ash dump; Table S5: The mesostructure parameters of the lower leaf of *N. ovata* from the natural forest community (P-1) and the fly ash dump (P-2).

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References

1. Chase, M.W.; Cameron, K.M.; Freudenstein, J.V.; Pridgeon, A.M.; Salazar, G.A.; van den Berg, C.; Schuiteman, A. An updated classification of Orchidaceae. *Bot. J. Linn. Soc.* **2015**, *177*, 151–174. [CrossRef]
2. Christenhusz, M.J.M.; Byng, J.W. The number of known plants species in the world and its annual increase. *Phytotaxa* **2016**, *261*, 201–217. [CrossRef]
3. Vakhrameeva, M.G.; Tatarenko, I.V. Ecological characteristics of orchids of European Part of Russia. *Acta Univ. Wratislav.* **2001**, *79*, 49–54.
4. Vakhrameeva, M.G.; Tatarenko, I.V.; Varlygina, T.I.; Torosyan, G.K.; Zagulskii, M.N. *Orchids of Russia and Adjacent Countries (within the Borders of the Former USSR)*; A.R.G. Gantner Verlag: Ruggell, Liechtenstein, 2008.
5. Kindlmann, P.; Jersakova, J. Floral display, reproductive success, and conservation of terrestrial orchids. *Selbyana* **2005**, *26*, 136–144. [CrossRef]
6. Sasamori, M.H.; Endres, D.J.; Droste, A. Asymbiotic culture of *Cattleya intermedia* Graham (Orchidaceae): The influence of macronutrient salts and sucrose concentrations on survival and development of plantlets. *Acta Bot. Bras.* **2015**, *29*, 292–298. [CrossRef]
7. Einzmann, H.J.R.; Schickenberg, N.; Zotz, G. Variation in root morphology of epiphytic orchids along small–scale and large–scale moisture gradients. *Acta Bot. Bras.* **2020**, *34*, 66–73. [CrossRef]
8. Brodmann, J.; Twele, R.; Francke, W.; Hölzler, G.; Zhang, Q.H.; Ayasse, M. Orchids mimic green–leaf volatiles to attract prey–hunting wasps for pollination. *Curr. Biol.* **2008**, *18*, 740–744. [CrossRef] [PubMed]
9. Brys, R.; Jacquemyn, H.; Hermy, M. Pollination efficiency and reproductive patterns in relation to local plant density, population size, and floral display in the rewarding *Listera ovata* (Orchidaceae). *Bot. J. Linn. Soc.* **2008**, *157*, 713–721. [CrossRef]
10. Jakubska–Busse, A.; Kadej, M. The pollination of Epipactis Zinn, 1757 (Orchidaceae) species in central Europe—the significance of chemical attractants, floral morphology and concomitant insects. *Acta Soc. Bot. Pol.* **2011**, *80*, 49–57. [CrossRef]
11. Ehlers, B.K.; Pedersen, H. Genetic variation in three species of Epipactis (Orchidaceae): Geographic scale and evolutionary inferences. *Biol. J. Linn. Soc.* **2000**, *69*, 411–430. [CrossRef]
12. Hens, H.; Jäkäläniemi, A.; Tali, K.; Efimov, P.; Kravchenko, A.V.; Kvist, L. Genetic structure of a regionally endangered orchid, the dark red helleborine (*Epipactis atrorubens*) at the edge of its distribution. *Genetica* **2017**, *145*, 209–221. [CrossRef]
13. Jurkiewicz, A.; Turnau, K.; Mesjasz–Przybylowicz, J.; Przybylowicz, W.; Godzik, B. Heavy metal localisation in mycorrhizas of *Epipactis atrorubens* (Hoffm.) Besser (Orchidaceae) from zink mine tailings. *Protoplasma* **2001**, *218*, 117–124. [CrossRef]
14. Shefferson, R.; Kull, T.; Tali, K. Mycorrhizal interactions of orchids colonizing Estonian mine tailings hills. *Am. J. Bot.* **2008**, *95*, 156–164. [CrossRef]
15. Tešitelová, T.; Tešitel, J.; Jersáková, J.; Říhová, G.; Selosse, M.S. Symbiotic germination capability of four Epipactis species (Orchidaceae) is broader than expected from adult ecology. *Am. J. Bot.* **2012**, *99*, 1020–1032. [CrossRef]
16. Zhang, S.; Yang, Y.; Li, J.; Qin, J.; Zhang, W.; Hu, H. Physiological diversity of orchids. *Plant Divers.* **2018**, *40*, 196–208. [CrossRef]
17. Swarts, D.N.; Dixon, W.D. Terrestrial orchid conservation in the age of extinction. *Ann. Bot.* **2009**, *104*, 543–556. [CrossRef] [PubMed]
18. Adamowski, W. Expansion of native Orchids in anthropogenous habitats. *Pol. Bot. Stud.* **2006**, *22*, 35–44.
19. Jermakowicz, E.; Brzosko, E. Demographic responses of boreal–montane orchid *Malaxis monophyllos* (L.) Sw. populations to contrasting environmental conditions. *Acta Soc. Bot. Pol.* **2016**, *85*, 1–17. [CrossRef]
20. Rewicz, A.; Bomanowska, A.; Shevera, M.; Kurowski, J.; Krason, K.; Zielinska, K. Cities and disturbed areas as man–made shelters for orchid communities. *Not. Bot. Horti Agrobot. Cluj Napoca* **2017**, *45*, 126–139. [CrossRef]
21. Agostini, A.; Caltagirone, C.; Caredda, A.; Cicatelli, A.; Cogoni, A.; Farci, D.; Guarino, F.; Garau, A.; Labra, M.; Lussu, M.; et al. Heavy metal tolerance of orchid populations growing on abandoned mine tailings: A case study in Sardinia Island (Italy). *Ecotoxicol. Environ. Saf.* **2020**, *189*, 110018. [CrossRef] [PubMed]
22. Filimonova, E.I.; Lukina, N.V.; Glazyrina, M.A. Orchidaceae in the technogenic ecosystems of the Urals. *Ecosystems* **2014**, *11*, 68–75. (In Russian)
23. Filimonova, E.I.; Lukina, N.V.; Glazyrina, M.A.; Borisova, G.G.; Maleva, M.G.; Chukina, N.V. Endangered orchid plant *Epipactis atrorubens* on serpentine and granite outcrops of Middle Urals, Russia: A comparative morphophysiological study. *AIP Conf. Proc.* **2019**, 040016. [CrossRef]
24. Filimonova, E.; Lukina, N.; Glazyrina, M.; Borisova, G.; Tripti; Kumar, A.; Maleva, M. A comparative study of *Epipactis atrorubens* in two different forest communities of the Middle Urals, Russia. *J. For. Res.* **2020**, *31*, 2111–2120. [CrossRef]
25. Kotilínek, M.; Těšitelová, T.; Jersáková, J. Biological Flora of the British Isles: *Neottia ovata*. *J. Ecol.* **2015**, *103*, 1354–1366. [CrossRef]

26. Vladimir Nikolaevich, B.; Nikolai Sergeevich, K. *Red Book of the Sverdlovsk Region: Animals, Plants, Mushrooms*; Mir LLC: Yekaterinburg, Russia, 2018. (In Russian)
27. Wiegand, K.M.A. Revision of the Genus Listera. *J. Torrey Bot. Soc.* **1899**, *26*, 157–171. [CrossRef]
28. Brzosko, E. The dynamics of Listera ovata populations on mineral islands in the Biebrza National Park. *Acta Soc. Bot. Pol.* **2002**, *71*, 243–251. [CrossRef]
29. Gajic´, G.; Djurdjevic´, L.; Kostic, O.; Jaric´, S.; Mitrovic´, M.; Pavlovic, P. Ecological potential of plants for phytoremediation and ecorestoration of fly ash deposits and mine wastes. *Front. Environ. Sci.* **2018**, *6*, 124. [CrossRef]
30. Pandey, V.C.; Prakash, P.; Bajpai, O.; Kumar, A.; Singh, N. Phytodiversity on fly ash deposits: Evaluation of naturally colonized species for sustainable phytorestoration. *Environ. Sci. Pollut. Res.* **2015**, *22*, 2776–2787. [CrossRef]
31. Filimonova, E.I.; Lukina, N.V.; Glazyrina, M.A.; Veselkin, D.V.; Chibrik, T.S.; Stephanovich, G.S. Morphological features of juvenile *Pinus sylvestris* L. on the ash dumps in the Middle Urals. *AIP Conf. Proc.* **2019**, 030008. [CrossRef]
32. Mokronosov, A.T. Mesotructure and functional activity of the photosynthetic apparatus. In *Mesotructure and Functional Activity of the Photosynthetic Apparatus*; Mokronosov, A.T., Ed.; Ural. Gos. Univ.: Sverdlovsk, Russia, 1978; pp. 5–15. (In Russian)
33. Mokronosov, A.T.; Gavrilenko, V.F.; Zhigalova, T.V. *Photosynthesis. Physiological and Environmental and Biochemical Aspects*; Academia: Moscow, Russia, 2006. (In Russian)
34. Ivanova, L.A.; P'yankov, V.I. Structural adaptation of the leaf mesophyll to shading. *Russ. J. Plant Physiol.* **2002**, *49*, 419–431. [CrossRef]
35. Ivanova, L.A. Adaptive features of leaf structure in plants of different ecological groups. *Russ. J. Ecol.* **2014**, *45*, 107–115. [CrossRef]
36. Terashima, I.; Miyazawa, S.; Hanba, Y.T. Why are sun leaves thicker than shade leaves?—Consideration based on analyses of CO_2 diffusion in the leaf. *J. Plant Res.* **2001**, *114*, 93–105. [CrossRef]
37. Slaton, M.R.; Smith, W.K. Mesophyll architecture and cell exposure to intercellular air space in alpine, desert, and forest species. *Int. J. Plant Sci.* **2002**, *163*, 937–948. [CrossRef]
38. Nekrasova, O.; Radchenko, T.; Filimonova, E.; Lukina, N.; Glazyrina, M.; Dergacheva, M.; Uchaev, A.; Betekhtina, A. Natural forest colonisation and soil formation on ash dump in southern taiga. *Folia For. Pol. Ser. A For.* **2020**, *62*, 306–316. [CrossRef]
39. Polley, J.R. Colorimetric determination of nitrogen in biological materials. *Anal. Chem.* **1954**, *26*, 1523–1524. [CrossRef]
40. Fiske, C.H.; Subbarow, Y. The colorimetric determination of phosphorus. *J. Biol. Chem.* **1925**, *66*, 375–400. [CrossRef]
41. Hellmuth, E.O. Measurement of Leaf Water Deficit with Particular Reference to the Whole Leaf Method. *J. Ecol.* **1970**, *58*, 409–417. [CrossRef]
42. Lichtenthaler, H.K. [34] Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. *Methods Enzymol.* **1987**, *148*, 350–382. [CrossRef]
43. Pandey, V.C.; Abhilashb, P.C.; Upadhyaya, R.N.; Tewari, D.D. Application of fly ash on the growth performance and translocation of toxic heavy metals within *Cajanus cajan* L.: Implication for safe utilization of fly ash for agricultural production. *J. Hazard. Mater.* **2009**, *166*, 255–259. [CrossRef]
44. Kabata–Pendias, A.; Mukherjee, A.B. *Trace Elements from Soil to Human*; Springer: Berlin/Heidelberg, Germany, 2007.
45. Marschner, H. *Mineral Nutrition of Higher Plants*, 2nd ed.; Academic Press: San Diego, CA, USA, 1995.
46. Hejcman, M.; Schellberg, J.; Pavlu, V. Dactylorhiza maculata, Platanthera bifolia and Listera ovata survive N application under P limitation. *Acta Oecol.* **2010**, *36*, 684–688. [CrossRef]
47. Van Arendonk, J.J.C.M.; Poorter, H. The chemical composition and anatomical structure of leaves of grass species differing in relative growth rate. *Plant Cell Environ.* **1994**, *17*, 963–970. [CrossRef]
48. Ashraf, M.; Harris, P.J.C. Photosynthesis under stressful environments: An overview. *Photosynthetica* **2013**, *51*, 163–190. [CrossRef]
49. Golovko, T.; Tabalenkova, G. Pigments and productivity of the crop plants. In *Photosynthetic Pigments: Chemical Structure, Biological Function and Ecology*; Golovko, T.K., Gruszecki, W.I., Prasad, M.N.V., Strzalka, K.J., Eds.; Komi Scientific Centre of the Ural Branch of the Russian Academy of Sciences: Syktyvkar, Russia, 2014; pp. 207–220.
50. Gruszecki, W.; Szymanska, R.; Fiedor, L. Carotenoids as photoprotectors. In *Photosynthetic Pigments: Chemical Structure, Biological Function and Ecology*; Golovko, T.K., Gruszecki, W.I., Prasad, M.N.V., Strzalka, K.J., Eds.; Komi Scientific Centre of the Ural Branch of the Russian Academy of Sciences: Syktyvkar, Russia, 2014; pp. 161–170.