Hacquetia 26: e2007-04 DOI: 10.3986/hacq-2027-0004

Elementome of *Orchis anthropophora* and *O. militaris* and their hybrid from the Réserve naturelle domaniale de Lanaye (Belgium)

Antony van der Ent[1], Ksenija Jakovljević[2], Mirko Salinitro[1], Vladan Djordjević[3]

Keywords: Biogeochemistry, Elementome, Hybrids, Orchids

Ključne besede: biogeokemija, elementom, križanci, orhideje

Abstract

The main objective of this study was to determine elemental profiles of *Orchis anthropophora*, *O. militaris* and their hybrid (*Orchis* × *spuria*) in order to deduce their adaptive responses within the hybrid zone in the Réserve naturelle domaniale de Lanaye. Foliar samples of orchids were collected and subjected to monochromatic X-ray fluorescence analysis for major and trace elements. While the macro-elements predominantly accumulated in the soil and leaf samples of *O. anthropophora*, the highest trace element concentrations were observed in *O. militaris*, with intermediate values for most elements analysed in the hybrid *O.* × *spuria*. This study is the first to investigate variation in accumulation between parental species and their hybrid, with intermediate inheritance observed in *Orchis* × *spuria*. Moreover, the analysed species confirmed exclusion as the most common strategy for coping with high prevailing elemental concentrations, as observed in previous studies.

Izvleček

Glavni cilj raziskave je bil ugotoviti elementni profil vrst *Orchis anthropophora*, *O. militaris* in njunega križanca (*Orchis* × *spuria*), s pomočjo katerih bi ugotovili njihove prilagoditvene odzive v hibrdinem območju rezervata Réserve naturelle domaniale de Lanaye. Zbrali smo vzorce listov orhidej in jih analizirali z monokromatsko rentgensko analizo, s čimer bi ugotovili sestavo makroelementov in elementov v sledovih. Makroelementi se akumulirajo predvsem v vzorcih tal in listov vrste *O. anthropophora*, vendar smo najvišje koncentracije elementov opazili pri vrsti *O. militaris.* Vmesne vrednosti smo dobili pri njunem križancu *O.* × *spuria.* Ta raziskava je prva, ki obravnava variabilnost akumulacije med starševskimi vrstami in njihovimi križanci z vmesnim dedovanjem, opazovanim pri *Orchis* × *spuria*. Poleg tega smo pri analiziranih vrstah potrdili, da je najpogostejša strategija za spoprijemanje s stresom zaradi težkih kovin izločanje, kar so ugotovili že v predhodnih raziskavah.

Corresponding author:
Ksenija Jakovljević
E-mail:
ksenija.jakovljevic@ibiss.bg.ac.rs
Received: 19. 6. 2026
Accepted: 15.10.2026

CC BY NC ND

1 Laboratory of Genetics, Wageningen University and Research, The Netherlands
2 Department of Ecology, Institute for Biological Research Siniša Stanković – National Institute of the Republic of Serbia, University of Belgrade, Serbia
3 Faculty of Biology, Institute of Botany and Botanical Garden, University of Belgrade, Serbia

Introduction

Understanding the elemental and ecophysiological profiles of plants is of great importance, both for the knowledge of their biology and ecology and from an application point of view, as these characteristics provide information on how plants adapt to different environmental conditions and define their ecological niches (Jakovljević et al., 2023; Tekman et al., 2025). Rare and endangered plants, such as terrestrial orchids, grow on different geological substrates and frequently in habitats that are disturbed or altered to some extent (De Agostini et al., 2020; Djordjević & Tsiftsis, 2022). Based on Grime's theory of population strategies (Grime, 1979), some authors categorise terrestrial orchids as stress tolerators (Dressler, 1981), while others place them between stress tolerators and ruderal plants (Hágsater & Dumont, 1996). This indicates that orchids can grow under certain stress and disturbance conditions. For example, some orchids have been found to grow on natural metalliferous habitats, such as ultramafics (Djordjević & Tsiftsis, 2019; Jakovljević et al., 2025), and even on anthroposols, *i.e.,* soils that have been heavily modified or constructed by human activities (*e.g.* mine waste deposits, fly ash and soils in the vicinity of roads) (Jurkiewicz et al., 2001; Shefferson et al., 2008; De Agostini et al., 2020; Maleva et al., 2022). In Poland, *Epipactis atrorubens* (Hoffm.) Besser, *E. helleborine* (L.) Crantz and *Dactylorhiza majalis* (Rchb.) P.F.Hunt & Summerh. were found on zinc mine tailings rich in Zn, Pb and Cd (Jurkiewicz et al., 2001), whereas *Dactylorhiza majalis* subsp. *baltica* (Klinge) H.Sund., *Epipactis atrorubens* and *Orchis militaris* L. were recorded at an old shale mine site in Estonia (Shefferson et al., 2008).

Although many orchids grow on various geological substrates, not many species have been studied regarding elemental uptake and accumulation. Furthermore, only a few studies have analysed in detail the relationship between elements in soil and individual orchid organs (van der Ent et al., 2015; Mikavica et al., 2023; Jakovljević et al., 2025). Recent research indicates that the exclusion strategy is the most common response to high concentrations of elements in the soil which may have an adverse effect on orchids (Račko et al., 2021; Maleva et al., 2022; Mikavica et al., 2023; Jakovljević et al., 2023, 2025). In general, there are two basic distribution patterns of macro- and trace elements in orchids; trace elements remain mainly in the underground organs, with only a small proportion transferred to the shoots, while macro-elements are mainly accumulated in leaves and inflorescences (Jakovljević et al., 2025). However, extreme substrate conditions are reflected in leaf chemistry, as in Rothschild's Slipper Orchid (*Paphiopedilum rothschildianum* (Rchb.f.) Stein), which occurs on ultramafic substrates in Kinabalu Park in Malaysia (van der Ent et al., 2015). Most terrestrial orchids are sensitive to increased nutrient levels in the soil, as these may disrupt mycorrhizal relationships (Dijk et al., 1997). A negative effect of soil fertilisation was observed in studies in the Netherlands and Flanders, where a significant decrease in the abundance of *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase was recorded as a result of soil enrichment with phosphorus (Silvertown et al., 1994). Hence, many studies focus on the content of nitrogen, phosphorus, potassium and carbon in the soil and in individual orchid organs and on the influence of these elements on the mycorrhizal relationships between orchids and fungi (Cameron et al., 2006; Hejcman et al., 2010; Zahn et al., 2023). Mycorrhizal fungi play an important role in protection against potentially toxic metals in orchids (Jurkiewicz et al., 2001; Herrera et al., 2018). In *Epipactis atrorubens*, in which Pb and Zn are accumulated in the roots, the metals are mainly stored in the fungal cell walls, probably in association with fungal pigments such as melanin (Jurkiewicz et al., 2001).

Orchis Tourn. ex L. is a genus of terrestrial orchids comprising of 23 species and 10 subspecies (POWO, 2025), distributed across Europe, temperate Asia and North Africa (Delforge, 2006; Kretzschmar et al., 2007). *Orchis anthropophora* (L.) All. is a Mediterranean-Atlantic species, found from England, Belgium and the Netherlands in the north to Morocco and Algeria in the south and from Spain and England in the west to Cyprus and Rhodes in the east (Delforge, 2006; Jacquemyn et al., 2011a; Fay & Taylor, 2015). It is widespread in western Europe, but rare in the eastern and northern parts of its range (Jacquemyn et al., 2011a). *Orchis militaris* is a Eurasian species distributed from south-eastern Sweden and Russia across central European countries to northern Spain, central Italy, Romania, Bulgaria, Greece and Turkey (Farrell, 1985). These two species have so-called "anthropomorphic" lips and belong to the *O. militaris* group (Tyteca & Klein, 2008, 2009). They occur in xerophilous and mesophilous grasslands, shrublands and open forests, mainly on calcareous substrates (Farrell, 1985; Delforge, 2006, Jacquemyn et al., 2011a; Fay & Taylor, 2015; Šabanović et al., 2023). While *O. militaris* is rare in the Mediterranean region and grows from lowlands to high mountains at altitudes up to 2200 m, *O. anthropophora* also occurs in typical Mediterranean habitats such as the gariga (Delforge, 2006; Jacquemyn et al., 2011a; Fay & Taylor, 2015). *Orchis* × *spuria* Rchb.f., their natural hybrid, is distributed in western, central and southern Europe (Kretzschmar et al., 2007; Jacquemyn et al., 2011a; POWO, 2025). It has been recorded in Belgium, Cyprus,

France, Germany, Italy, the Netherlands, Spain and Switzerland (Kull et al., 2016; POWO, 2025). Both species and the hybrid are protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES Secretariat, United Nations Environment Programme (UNEP), 2023). The IUCN threat status of *O. anthropophora* ranges from "Least Concern" (in France and Spain) to "Regionally Extinct" in Cyprus, while the IUCN threat status of *O. militaris* in Europe ranges from "Least Concern" (in Spain, Sweden, France, Moldova and Hungary) to "Regionally Extinct" in Denmark (Kull et al., 2016).

The Sint-Pietersberg (Mount St Peter, Montagne St Pierre) is an area that extends across the border between Belgium (Wallonia and a small part of Flanders) and the Netherlands (Limburg province). It is a limestone ridge approximately 15 kilometres long and several kilometres wide, straddling the Dutch Belgian border south of Maastricht, shaped by the rivers Maas and Jeker (Kreutz et al., 2017). Owing to its calcareous substrates and specific climate, the area is notable for its high diversity of vascular flora, with a particularly significant presence of orchid taxa, whose abundance and habitat characteristics have been studied extensively over a long time (Kreutz, 1986, 1994; Kreutz et al., 2017; Verhart & Kreutz, 2021; Mol & Kreutz, 2024). A review of the orchid flora revealed the presence of approximately 30 orchid taxa and several orchid hybrids (Kreutz et al., 2017), with orchids occurring in both the Dutch and Belgian parts of Sint-Pietersberg, and high orchid species richness and population sizes in localities on both sides of the Albert Canal. The area contains numerous habitats – limestone grasslands, heath grasslands, wet meadows, roadsides, deciduous forests and shrubs – suitable for orchids (Kreutz et al., 2017). One of the most important reserves here is the Lanaye Orchid Réserve, located in Wallonia, Belgium, within the municipality of Visé, near the village of Lanaye.

Studies on hybrids and their parental species in the genus *Orchis* mainly focus on morphological and genetic traits (Bersweden et al., 2021), the spatial distribution of species in relation to mycorrhizal fungi (Jacquemyn et al., 2012), and mycorrhizal associations and reproductive isolation in related species (Jacquemyn et al., 2011b). In sympatric orchid populations, such as those in the Lanaye Orchid Réserve, hybridisation between parental taxa is common phenomenon and represents a substantial source of genetic variation (Hartvig et al., 2026). Although differences in elemental profiles may occur, to date, no comparative studies on the elemental composition of leaves in closely related *Orchis* species and their hybrids from natural habitats have yet been conducted. Therefore, the main objective of this study was to determine the elemental profiles of *O. anthropophora*, *O. militaris* and their hybrid (*Orchis* × *spuria*) to infer their adaptive responses within the hybrid zone in the Réserve naturelle domaniale de Lanaye (Belgium).

Materials and methods

Collection of samples in the field and processing for elemental analysis

Very small (<0.5 g) foliar samples of *O. anthropophora* (three samples), *O. militaris* (nine samples) and the hybrid (nine samples), together with their rhizosphere soil (<5 g), were collected in the Réserve naturelle domaniale de Lanaye, Belgium (N 50.78056°, E 05.68425°, alt 70 m a.s.l.) (Figures 1–3), in *Rubus fruticosus-Holcus lanatus* underscrub, in the hybrid zone where the two parental species grow in sympatry.



Figure 1: Lanaye Orchid Réserve in Belgium, showing the habitat of *Orchis militaris*, *O. anthropophora* and their hybrid *O.* × *spuria*.
Slika 1: Rezervat orhidej Lanaye v Belgiji, rastišče vrst *Orchis militaris*, *O. anthropophora* in njunega križanca *O.* × *spuria*.

The population of *O. militaris* was estimated at 150 to 200 individuals, much larger compared with *O. anthropophora* (four individuals) and the hybrid *O.* × *spuria* (15–20 individuals). The foliar samples were dried for 48 hrs at 60°C in a drying oven and then ground to a fine powder (<200 µm) using an impact mill (IKA TubeMill 100 Control). The soil samples were sieved to <1000 mm using a stainless-steel mesh screen (Royal Eijkelkamp Sieve Set Ø 200 mm).

Elemental analysis of plant and soil material samples

The powdered samples were placed in custom XRF sample holders and covered with 6.0 µm polypropylene thin film (Chemplex Industries Inc.) for XRF analysis. The XRF analysis was performed using a Z-Spec JP500 instrument (Z-Spec Inc.) in 'Plant' mode for the plant powdered material and in 'Soil' mode for the soil samples (Zhang et al., 2026). This instrument uses monochromatic X-ray fluorescence excitation at 17.48 keV to analyse elements from Z = 13 (Al) to Z = 40 (Zr) on the K-lines and up to Z = 92 (U) on the L-lines with optimum sensitivity for elements Cu–Se and Hg-Tl-Pb with limits of detection ranging from 0.009 to 0.025 mg $kg^{-1.}$ Two certified standards were included for the plant material analysis (NIST SRM 1570a Trace elements in spinach leaves and NIST SRM 1573a Tomato leaves), and two certified standards were included for the soil analysis (NIST SRM 2702, marine sediments and NIST SRM 2709a San Joaquin Soil). The recovery was between the 95% and 105% for each of the elements tested.



Figure 2: *Orchis militaris* (a) and *O. anthropophora* (b) *in situ* at the Lanaye Réserve, Belgium.
Slika 2: *Orchis militaris* (a) in *O. anthropophora* (b) *in situ* v rezervatu Lanaye, Belgija.



Figure 3: *Orchis* × *spuria* (*Orchis anthropophora* × *O. militaris*) *in situ* at the Lanaye Réserve, Belgium.
Slika 3: *Orchis* × *spuria* (*Orchis anthropophora* × *O. militaris*) *in situ* v rezervatu Lanaye, Belgija.

Statistical analyses

Elemental concentrations in the soil and leaves were presented as means and standard deviations, with the Kruskal-Wallis H test, followed by Dunn's test for pair-wise multiple comparisons ($p < 0.05$) used to determine significant differences between samples. To reduce data dimensionality, principal component analysis was used, visualised with 'dplyr' version 1.1.4., 'ggplot2' version 4.0.1. and 'ggrepel' version 0.9.6. All statistical analyses were performed in R (v. 4.3.2) (R Core Team, 2025).

Results

Elemental concentrations in soil samples from the three orchids analysed are presented in Table 1. The *O. anthropophora* samples were characterised by the highest mean macro-elemental concentrations, including Ca and P, while trace elements (Cr, Cu, Mn, Ni, Pb, Zn) were the most abundant in samples of *O.* × *spuria*. The range of values, however, often deviated from this pattern, as observed for Ca, Cl, Cr, P, Sr, and Zn. For most of the elements analysed, the soil samples of *O. militaris* showed intermediate values.

Table 1: Macro- and trace elemental concentrations in the soil samples of *Orchis militaris* (n = 9), *O. anthropophora* (n = 3) and *O.* × *spuria* (n = 9) from the Lanaye Réserve, Belgium (given in mg kg^{-1} as the mean value and SD).
Tabela 1: Koncentracije makroelementov in elementov v sledovih v vzorcih tal pri vrstah *Orchis militaris* (n = 9), *O. anthropophora* (n = 3) in *O.* × *spuria* (n = 9) iz rezervata Lanaye, Belgija (kot povprečna vrednost in SD v mg kg^{-1}).

		Orchis anthropophora	*Orchis militaris*	*Orchis* × *spuria*
As	mean	7.57 ± 8.4	2.47 ± 0.895	3.41 ± 1.36
	range	2.62–17.3	1.11–3.91	1.55–5.39
Ca	mean	337,000 ± 26,700	293,000 ± 74,300	255,000 ± 105,000
	range	319,000–368,000	129,000–382,000	111,000–392,000
Cl	mean	993 ± 69.3	854 ± 257	775 ± 316
	range	953–1070	299–1110	360–1290
Co	mean	0.132 ± 0.229	1.11 ± 2.75	0.406 ± 0.860
	range	0–0.397	0–8.29	0–2.41
Cr	mean	19.6 ± 1.12	37.2 ± 13.9	41.4 ± 21.3
	range	18.6–20.8	20.7–61.9	19.7–73.3
Cu	mean	2.89 ± 2.04	3.53 ± 3.82	6.64 ± 4.27
	range	0.541–4.15	0–9.01	0–11.4
Fe	mean	19,000 ± 702	23,100 ± 2060	24,100 ± 4320
	range	18,500–19,800	20,800–26,800	18,100–28,200
K	mean	2120 ± 443	4930 ± 3460	6960 ± 4870
	range	1690–2570	1950–13000	2200–13,300
Mn	mean	257 ± 33.9	308 ± 36.2	322 ± 54.2
	range	227–294	257–362	230–396
Ni	mean	8.38 ± 2.7	9.71 ± 4.6	12.3 ± 4.76
	range	5.74–11.1	3.81–19.2	5.79–18.7
P	mean	19,800 ± 1150	17,000 ± 5310	14,800 ± 7550
	range	18,500–20,600	5730–23,600	3600–25,300
Pb	mean	24.4 ± 4.05	25.0 ± 3.78	28.9 ± 4.98
	range	19.7–26.9	20.5–31.0	21.7–34.6
Rb	mean	8.26 ± 2.61	19.1 ± 14.5	31.2 ± 19.4
	range	6.28–11.2	6.20–54.2	8.18–60.8
S	mean	< LOD	71.2 ± 214	108 ± 213
	range	< LOD	0–641	0–572
Si	mean	4660 ± 4230	50,000 ± 53,000	77,700 ± 79,400
	range	0–8260	0–171,000	0–174,000
Sr	mean	609 ± 12.5	510 ± 128	434 ± 200
	range	597–622	212–632	177–667
Ti	mean	1230 ± 127	2160 ± 906	2330 ± 980
	range	1120–1370	1370–3600	1210–3750
Zn	mean	125 ± 36.7	168 ± 50.1	173 ± 47.1
	range	98.5–167	110–249	104–243

* No statistically significant differences between concentrations were detected (values not shown).

Table 2: Elemental concentrations in the leaf samples of *Orchis militaris* (n = 9), *O. anthropophora* (n = 3) and *O.* × *spuria* (n = 9) from the Lanaye Réserve, Belgium (given in mg kg^{-1} as the mean value and SD).
Tabela 2: Koncentracije elementov v vzorcih listov vrst *Orchis militaris* (n = 9), *O. anthropophora* (n = 3) in *O.* × *spuria* (n = 9) iz rezervata Lanaye, Belgija (kot povprečna vrednost in SD v mg kg^{-1}).

		Orchis anthropophora	*Orchis militaris*	*Orchis* × *spuria*
As	mean	0.066 ± 0.114	0.043 ± 0.072	0.038 ± 0.073
	range	0–0.198	0–0.202	0–0.21
Br	mean	0.847 ± 0.125	2.42 ± 0.633	1.89 ± 0.482
	range	0.718–0.967	1.49–3.55	1.14–2.41
Ca	mean	9540 ± 1540	9070 ± 2320	9380 ± 1800
	range	7980–11,100	6440–14,000	7490–12,000
Cl	mean	515 ± 293	794 ± 326	731 ± 479
	range	341–853	358–1220	129–1400
Co	mean	0.504 ± 0.11	0.523 ± 0.763	0.810 ± 0.425
	range	0.381–0.586	0–2.63	0.225–1.29
Cr	mean	0.900 ± 0.739	0.042 ± 0.090	0.258 ± 0.443
	range	0.08–1.51	0–0.253	0–1.26
Cu	mean	5.89 ± 0.741	7.12 ± 1.09	6.92 ± 0.875
	range	5.05–6.45	5.68–8.73	5.85–7.98
Fe	mean	104 ± 7.12	102 ± 62.8	124 ± 38.3
	range	95.6–109	55.3–274	65.6–167
K	mean	9570 ± 2640	16400 ± 2910	14200 ± 4020
	range	6700–11,900	12100–21300	7810–19,600
Mn	mean	11.6 ± 1.81	17.4 ± 2.86	16.6 ± 3.22
	range	9.64–13.2	12.3–21.1	13.2–23.1
Ni	mean	0.727 ± 0.312	0.614 ± 0.195	0.503 ± 0.199
	range	0.489–1.08	0.416–0.956	0.275–0.732
P	mean	1820 ± 312	1780 ± 522	1440 ± 423
	range	1470–2080	1240–2970	778–1800
Rb	mean	17.4 ± 5.19	18 ± 12	13.2 ± 8.24
	range	11.5–20.9	4.93–39.8	4.67–24
S	mean	1110 ± 95.6	1370 ± 238	1280 ± 292
	range	1040–1220	991–1820	805–1580
Se	mean	0.188 ± 0.105	0.163 ± 0.101	0.06 ± 0.037
	range	0.068–0.265	0–0.302	0–0.118
Si	mean	273 ± 237	272 ± 705	366 ± 490
	range	0–415	0–2230	0–1300
Sr	mean	14.3 ± 1.79	11.7 ± 2.69	13.9 ± 2.82
	range	12.2–15.3	8.63–17.7	9.80–16.8
Zn	mean	28.4 ± 4.17	33.1 ± 10.7	31.0 ± 7.45
	range	23.8–31.9	19.7–53.5	23.3–43.3

* No statistically significant differences between concentrations were detected (values not shown).

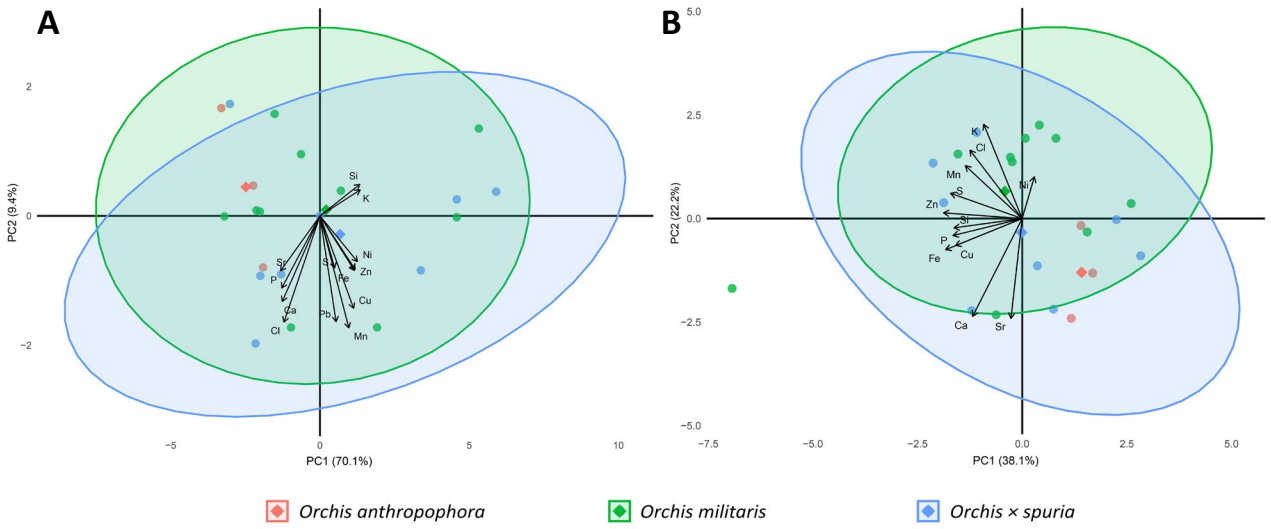


Figure 4: Principal component analysis of the elemental composition of a) soil and b) leaf samples of *Orchis anthropophora*, *O. militaris* and their hybrid *O.* × *spuria*.
Slika 4: Analiza glavnih komponent sestave vzorcev elementov a) tal in b) listov vrst *Orchis anthropophora*, *O. militaris* in njunega križanca *O.* × *spuria*.

Similar to the soil, calcium and phosphorus concentrations were highest in *O. anthropophora* leaf samples, whereas potassium was lowest in this taxon (Table 2). Potassium accumulated most strongly in *O. militaris*, as did Cu and Zn. Intermediate concentrations for most elements analysed, were observed in the hybrid *O.* × *spuria*. The principal component analysis indicates distinctions among the taxa analysed, in both soil and leaf samples, with the greatest differences between the parental taxa and an intermediate position for their hybrid *O.* × *spuria* (Figure 4). Iron, zinc and copper in the leaves contributed most to the first principal component (PC1), as indicated by the highest, though relatively uniform loadings. In the soil samples, high loadings on PC1 were also found for K, Si and Ni.

Discussion

Calcareous substrates, such as those in the Réserve naturelle domaniale de Lanaye, are considered the most important for terrestrial orchids in Europe (Landi et al., 2009; Djordjević & Tsiftsis, 2022), with *O. anthropophora* and *O. militaris* known to grow exclusively or predominantly on this type of substrate (Djordjević & Tsiftsis, 2022). High concentrations of Ca^{2+} (nearly 400,000 mg kg^{-1} of total Ca) reduce the availability of elements such as Fe and Mn, as well as P, due to conversion to insoluble calcium phosphate (Dijk et al., 1997). An imbalance was also found between calcium and potassium, as frequently reported in the literature (Wahba et al., 2019), with the lowest concentrations of K observed in soil samples of *O. anthropophora*, which had the highest calcium content. In addition, calcareous soils are generally not considered a source of trace elements, with concentrations well-below the toxicity limits (Krämer, 2010), and only minor variations among the samples. Albeit subtle, variations in elemental concentrations at the microhabitat level affected the occurrence patterns of analysed species. The mean values place soil samples of *O. anthropophora* and *O.* × *spuria* at opposite ends, with higher concentrations of nearly all elements analysed in the rhizosphere of the hybrid taxon.

The macro-elemental distribution observed in the soil samples of *O. anthropophora* was reflected in the leaves, with a less clearly defined gradient in *O. militaris* and the hybrid taxon. The association of *O. anthropophora* with higher calcium content in the soil indicates a greater degree of specialisation towards carbonate substrates, as emphasised in previous studies (Jacquemyn et al., 2011; Fay & Taylor, 2015). Intensive accumulation of macro-elements in the above-ground tissues is a known pattern in orchids (Maleva et al., 2022; Jakovljević et al., 2023, 2025), consistent with their role in enzyme activation and regulation, chlorophyll production, flower initiation, and seed production (Marschner, 2012; Guo et al., 2023), and with the fact that orchids primarily invest in reproduction, making them more r-selected than K-selected species, despite their perennial nature (Pianka, 1970).

The concentrations of Ca, K, and P varied among species, including those from carbonate substrates, with the highest values observed in *Neottia ovata* (L.) Hartm., from natural and fly-ash sites in Russia (Maleva et al., 2021). In contrast to macro-elements, the tolerance strategy of orchids relies largely on the exclusion of trace elements (Račko et al., 2021; Maleva et al., 2022; Jakovljević et al., 2023, 2025), with no differences observed in hyperaccumulation capacity between metalliferous and non-metalliferous sites. Exclusion was evident even in calcareous substrates where, due to high pH values, extractable trace element concentrations in the soil and metal stress are negligible (Jakovljević et al., 2023). Soil concentrations in Réserve naturelle domaniale de Lanaye, while low, still resulted in orchid-specific trends. With the exception of Co, Cr, and Ni, ultramafic-specific elements (Reeves et al., 1999; Echevarria, 2021), strongly depleted in carbonate soil (Najafi-Ghiri et al., 2013), trace element concentrations followed a gradient, with the highest concentrations in *O. militaris*, the lowest in *O. anthropophora*, and intermediate values in their hybrid – *O.* × *spuria*. This pattern was also evident from the PCA results, which indicated separation between the parental orchids, with the hybrid taxa occupying an intermediate position. Considering the slight variability in substrate characteristics, the differences in elemental accumulation may have resulted from genetic heterogeneity and represent incomplete dominance or intermediate inheritance (Strome et al., 2024), where neither allele is completely dominant, and the hybrid genotype produces a phenotype intermediate between the parental phenotypes. In species such as *Arabidopsis kamchatica* (Fisch. ex DC.) K.Shimizu & Kudoh, however, the hybridisation process resulted in allopolyploidy, a mechanism that may lead to increased tolerance compared to the parental *A. halleri* (L.) O'Kane & Al-Shehbaz and *A. lyrata* (L.) O'Kane & Al-Shehbaz, and more efficient accumulation or colonisation of new habitats (Paape et al., 2020). Although introgression predominates, allopolyploidy in orchids is also not unknown, and is considered a main mechanism of speciation in *Dactylorhiza* Neck. ex Nevski (Hedrén, 1996).

Conclusions

The results of this study highlight the intermediate element content of hybrid leaves. Until now, it has been known that hybrid individuals, both morphologically and genetically (Bersweden et al., 2021), show characteristics intermediate between their parental species, while their elemental profiles have been overlooked. Further research should examine the detailed ecophysiology of the parental species and their hybrids, and their responses in relation to the different habitats and geological substrates on which they grow. Moreover, establishing monitoring of the studied species in the Réserve naturelle domaniale de Lanaye would contribute to species conservation as the habitat conditions raise concerns about overgrowth, specifically succession and increasing encroachment of scrub and woody vegetation at the expense of grasslands, probably related to increased nutrient content in the soil and greater plant biomass production.

Acknowledgements

We thank the Service public de Wallonie (SPW), Département Nature et Forêts (DNF) of the Belgian Government for provision of the collection permit (déro 2025-RS-l 5 - Orchidées). We thank Jordy Litjens for analysing the plant and soil samples with the Z-Spec instrument.

Author Contributions

AvdE, KJ, MS and VDj collected the samples in the field. AvdE prepared the samples for analysis. AvdE, KJ and VDj wrote the first draft of the manuscript. All authors reviewed and approved the final draft of the manuscript.

Funding

This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Grant Numbers 451-03-33/2026-03/200007 and 451-03-33/2026-03/200178).

Research Data Availability

The data that support this study will be shared upon reasonable request to the corresponding author.

Conflict of Interest

The authors declare no conflicts of interest relevant to the content of this manuscript.

References

Bersweden, L., Viruel, J., Schatz, B., Harland, J., Gargiulo, R., Cowan, R. S., Calevo, J., Juan, A., Clarkson, J. J., Leitch, A. R., & Fay, M. F. (2021). Microsatellites and petal morphology reveal new patterns of admixture in *Orchis* hybrid zones. *American Journal of Botany*, *108*(8), 1388–1404. https://doi.org/10.1002/ajb2.1710

Cameron, D. D., Leake, J. R., & Read, D. J. (2006). Mutualistic mycorrhiza in orchids: evidence from plant–fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*.

New Phytologist, *171*(2), 405–416. https://doi.org/10.1111/j.1469-8137.2006.01767.x

CITES Secretariat, United Nations Environment Programme (UNEP) (2023). Convention on International Trade in Endangered Species of Wild Fauna and Flora: Appendices I, II and III: valid from 25 November 2023. Geneva: CITES Secretariat. Available at: https://bvearmb.do/handle/123456789/3777. [Accessed 2 September 2025]

De Agostini, A., Caltagirone, C., Caredda, A., Cicatelli, A., Cogoni, A., Farci, D., Guarino, F., Garau, A., Labra, M., Lussu, M., Piano, D., Sanna, C., Tommasi, N., Vacca, A., & Cortis, P. (2020). Heavy metal tolerance of orchid populations growing on abandoned mine tailings: A case study in Sardinia Island (Italy). *Ecotoxicology and Environmental Safety*, *189*, 110018. https://doi.org/10.1016/j.ecoenv.2019.110018

Delforge, P. (2006). *Orchids of Europe, North Africa and the Middle East*. A. & C. Black.

Dijk, E., Willems, J. H., & van Andel, J. (1997). Nutrient responses as a key factor to the ecology of orchid species. *Acta Botanica Neerlandica*, *46*(4), 339–363.

Djordjević, V., & Tsiftsis, S. (2019). Patterns of orchid species richness and composition in relation to geological substrates. *Wulfenia*, *26*, 1–21.

Djordjević, V., & Tsiftsis, S. (2022). The role of ecological factors in distribution and abundance of terrestrial orchids. In A. Rosmala & N. Hardarani (Eds.), *Orchids phytochemistry, biology and horticulture: fundamentals and applications* (pp. 3–72). Springer International Publishing.

Dressler, R. L. (1981). *The orchids. Natural history and classification*. Horvard University Press.

Echevarria, G. (2021). Genesis and behaviour of ultramafic soils and consequences for nickel biogeochemistry. In A. van der Ent, A. J. M. Baker, G. Echevarria, M. O. Simonnot, & J. L. Morel (Eds.), *Agromining: Farming for metals: Extracting unconventional resources using plants* (pp. 215–238). Springer International Publishing.

Farrell, L. (1985). Biological flora of the British Isles: *Orchis militaris* L. *Journal of Ecology*, *73*, 1041–1053.

Fay, M. F., & Taylor, I. (2015). 805. *Orchis anthropophora*: Orchidaceae. *Curtis's Botanical Magazine*, *32*(1), 63–71. https://doi.org/10.1111/curt.12096

Grime, J. P. (2006). *Plant strategies, vegetation processes, and ecosystem properties*. John Wiley & Sons.

Guo, H., Dong, Q., Li, S., Cha, X., Sun, L., Duan, H., Li, S., Jin, Y., & Zhang, M. (2023). Effects of exogenous calcium on growth, chlorophyll fluorescence characteristics and antioxidant system of *Fraxinus malacophylla* seedlings. *Plant Physiology and Biochemistry, 201*, 107860. https://doi.org/10.1016/j.plaphy.2023.107860

Hagsater, E., Dumont, V., & Pridgeon, A. M. (Eds.). (1996). *Orchids: status survey and conservation action plan (Vol. 28)*. IUCN.

Hartvig, I., Ligon, J., Bünner, M. S., Evans, S. A., Eserman, L., Whigham, D. F., Coffey, M. K., & McCormick, M. K. (2026). Conservation genomics of the fringed orchids (*Platanthera*) in the Eastern US–species boundaries and genetic structure across landscapes. *Biodiversity and Conservation*, *35*(2), 45. https://doi.org/10.1007/s10531-025-03199-3

Hedrén, M. (1996). Genetic differentiation, polyploidization and hybridization in northern European *Dactylorhiza* (Orchidaceae): evidence from allozyme markers. *Plant Systematics and Evolution*, *201*(1), 31–55. https://doi.org/10.1007/BF00989050

Hejcman, M., Schellberg, J., & Pavlů, V. (2010). *Dactylorhiza maculata*, *Platanthera bifolia* and *Listera ovata* survive N application under P limitation. *Acta Oecologica*, *36*(6), 684–688. https://doi.org/10.1016/j.actao.2010.09.001

Herrera, H., Valadares, R., Oliveira, G., Fuentes, A., Almonacid, L., do Nascimento, S. V., Bashan, Y., & Arriagada, C. (2018). Adaptation and tolerance mechanisms developed by mycorrhizal *Bipinnula fimbriata* plantlets (Orchidaceae) in a heavy metal-polluted ecosystem. *Mycorrhiza*, *28*(7), 651–663. https://doi.org/10.1007/s00572-018-0858-4

Jacquemyn, H., Brys, R., Cammue, B. P., Honnay, O., & Lievens, B. (2011b). Mycorrhizal associations and reproductive isolation in three closely related *Orchis* species. *Annals of Botany*, *107*(3), 347–356. https://doi.org/10.1093/aob/mcq248.

Jacquemyn, H., Brys, R., Honnay, O., Roldán-Ruiz, I., Lievens, B., & Wiegand, T. (2012). Nonrandom spatial structuring of orchids in a hybrid zone of three *Orchis* species. *New Phytologist*, *193*(2), 454–464. https://doi.org/10.1111/j.1469-8137.2011.03913.x

Jacquemyn, H., Brys, R., & Hutchings, M. J. (2011a). Biological flora of the British Isles: *Orchis anthropophora* (L.) All. (*Aceras anthropophorum* (L.) WT Aiton). *Journal of Ecology*, *99*(6), 1551–1565. https://doi.org/10.1111/j.1365-2745.2011.01897.x

Jakovljević, K., Mišljenović, T., Djordjević, V., van der Ent, A., Ćosić, M., Andrejić, G., & Šinžar-Sekulić, J. (2023). Elemental and ecophysiological profiles of orchid *Dactylorhiza sambucina* show distinct responses to contrasting geological substrates. *Flora*, *303*, 152276. https://doi.org/10.1016/j.flora.2023.152276

Jakovljević, K., Ranđelović, D., Mutić, J., Mišljenović, T., Vujičić, M., van der Ent, A., & Djordjević, V. (2025). The edaphic factor and orchids: *Gymnadenia conopsea* from contrasting geologies in the Central Balkans. *Planta*, *261*(2), 38. https://doi.org/10.1007/s00425-025-04612-5

Jurkiewicz, A., Turnau, K., Mesjasz-Przybyłowicz, J., Przybyłowicz, W., & Godzik, B. (2001). Heavy metal localisation in mycorrhizas of *Epipactis atrorubens* (Hoffm.) Besser (Orchidaceae) from zinc mine tailings. *Protoplasma*, *218*(3), 117–124. https://doi.org/10.1007/BF01306601

Krämer, U. (2010). Metal hyperaccumulation in plants. *Annual Review of Plant Biology, 61*(1), 517–534. https://doi.org/10.1146/annurev-arplant-042809-112156.

Kretzschmar, H., Eccarius, W., & Dietrich, H. (2007). *The orchid genera Anacamptis, Orchis and Neotinea: phylogeny, taxonomy, morphology, biology, distribution, ecology and hybridisation*. EchinoMedia Verlag.

Kreutz, C. A. J. (1986). De orchideeënpopulaties op het Belgisch deel van de St. Pietersberg. *Natuurhistorisch Maandblad*, *75*(5), 87–93.

Kreutz, C. A. J. (1994). Bijzondere vondsten op het Belgische gedeelte van de St. Pietersberg. *Orchideeën*, *56*(3), 20–21.

Kreutz, C. A. J., Harle, N., & Lejeune, M. (2017). *Orchideeën van de Sint-Pieters berg. Een historisch en actueel overzicht*. Stichting Natuurpublicaties Limburg.

Kull, T., Selgis, U., Pecina, M. V., Metsare, M., Ilves, A., Tali, K., Sepp, K., Kull, K., & Shefferson, R. P. (2016). Factors influencing IUCN threat levels to orchids across Europe on the basis of national red lists. *Ecology and Evolution*, *6*(17), 6245–6265. https://doi.org/10.1002/ece3.2363

Landi, M., Frignani, F., Lazzeri, C., & Angiolini, C. (2009). Abundance of orchids on calcareous grasslands in relation to community species, environmental, and vegetational conditions. Russian *Journal of Ecology*, *40*(7), 486–494. https://doi.org/10.1134/S1067413609070066

Maleva, M., Borisova, G., Filimonova, E., Lukina, N., Chukina, N., Ermoshin, A., Tugbaeva, A., & Voropaeva, O. (2022). Adaptive redox reactions promote naturalization of rare orchid *Epipactis atrorubens* on serpentine dumps post asbestos mining. *Horticulturae*, *8*(7), 603. https://doi.org/10.3390/horticulturae8070603

Marschner, H. (Ed.). (2011). *Marschner's mineral nutrition of higher plants*. Academic Press.

Mikavica, I., Ranđelović, D., Djordjević, V., Rakić, T., Gajić, G., & Mutić, J. (2023). Concentration and mobility of trace elements (Li, Ba, Sr, Ag, Hg, B) and macronutrients (Ca, Mg, K) in soil-orchid system on different bedrock types. *Environmental Science and Pollution Research*, *30*(1), 979–995. https://doi.org/10.1007/s11356-022-22110-z

Mol, D. B. J. P., & Kreutz, K. C. A. J. (2024). Orchideeën in een bijzonder kalkgrasland in het Heuvelland. *Natuurhistorisch Maandblad*, *113*(3), 73–84.

Najafi-Ghiri, M., Ghasemi-Fasaei, R., & Farrokhnejad, E. (2013). Factors affecting micronutrient availability in calcareous soils of Southern Iran. *Arid Land Research and Management*, *27*(3), 203–215. https://doi.org/10.1080/03650340.2011.573788

Paape, T., Akiyama, R., Cereghetti, T., Onda, Y., Hirao, A. S., Kenta, T., & Shimizu, K. K. (2020). Experimental and field data support range expansion in an allopolyploid *Arabidopsis* owing to parental legacy of heavy metal hyperaccumulation. *Frontiers in Genetics*, *11*, 565854. https://doi.org/10.3389/fgene.2020.565854

Pianka, E. R. (1970). On r-and K-selection. *The American Naturalist*, *104*(940), 592–597. https://doi.org/10.1086/282697

POWO. (2025). Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. Available at: http://www.plantsoftheworldonline.org/ [Accessed 2 September 2025].

R Core Team. (2025). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria (URL). https://www.R-project.org/

Račko, M., Ozdín, D., Kučerová, G., Jurkovič, Ľ., & Vaculík, M. (2021). Occurrence and uptake of heavy metals by selected terrestrial orchids in extreme conditions of initial soils on previous mining sites. *Biologia*, *76*(7), 2113–2122. https://doi.org/10.2478/s11756-020-00619-3

Reeves, R. D., Baker, A. J. M., Borhidi, A., & Berazain, R. (1999). Nickel hyperaccumulation in the serpentine flora of Cuba. *Annals of Botany*, *83*(1), 29–38. https://doi.org/10.1006/anbo.1998.0786.

Shefferson, R. P., Kull, T., & Tali, K. (2008). Mycorrhizal interactions of orchids colonizing Estonian mine tailings hills. *American Journal of Botany*, *95*(2), 156–164. https://doi.org/10.3732/ajb.95.2.156

Silvertown, J., Wells, D. A., Gillman, M., Dodd, M. E., Robertson, H., & Lakhani, K. H. (1994). Short-term effects and long-term after-effects of fertilizer application on the flowering population of green-winged orchid *Orchis morio*. *Biological Conservation*, *69*(2), 191–197. https://doi.org/10.1016/0006-3207(94)90059-0

Strome, S., Bhalla, N., Kamakaka, R., Sharma, U., & Sullivan, W. (2024). Clarifying Mendelian vs non-Mendelian inheritance. *Genetics*, *227*(3), iyae078. https://doi.org/10.1093/genetics/iyae078

Šabanović, E., Djordjević, V., Milanović, Đ., Gocić, D. J., Bektić, S., Huseinović, S., & Ranđelović, V. (2023). *Orchis anthropophora* (L.) All. (Orchidaceae), a confirmed species for the flora of Bosnia and Herzegovina. *Biologica Nyssana*, *14*(1), 7–13. https://doi.org10.5281/zenodo.8027050

Tekman, E., Asgarlı, T., Yuca, H., Atila, A., Çeçen, Ö., & Karakaya, S. (2025). Exploring quantitative biological major, trace, and ultratrace elements composition and qualitative primary-secondary metabolites in Lamiaceae medicinal plants from Turkey. *Biological Trace Element Research*, *203*(2), 1188–1201. https://doi.org/10.1007/s12011-024-04219-z

Tyteca, D., & Klein, E. (2008). Genes, morphology and biology–The systematics of Orchidinae revisited. *Journal of Europaischer Orchideen*, *40*(3), 501–544.

van der Ent, A., van Vugt, R., & Wellinga, S. (2015). Ecology of *Paphiopedilum rothschildianum* at the type locality in Kinabalu Park (Sabah, Malaysia). *Biodiversity and Conservation*, *24*(7), 1641–1656. https://doi.org/10.1007/s10531-015-0881-0

Verhart, F., & Kreutz, K. (2021). Orchideeën koloniseren jonge Zuid-Limburgse loofbosjes. *Natuurhistorisch Maandblad*, *110*(10), 230–242.

Wahba, M., Fawkia, L. A. B. I. B., & Zaghloul, A. (2019). Management of calcareous soils in arid region. *International Journal of Environmental Pollution and Environmental Modelling*, *2*(5), 248–258.

Zahn, F. E., Söll, E., Chapin, T. K., Wang, D., Gomes, S. I., Hynson, N. A., Pausch, J., & Gebauer, G. (2023). Novel insights into orchid mycorrhiza functioning from stable isotope signatures of fungal pelotons. *New Phytologist*, *239*(4), 1449–1463. https://doi.org/10.1111/nph.18991

Zhang, C., Charrois, L., Jacquet, J., Sirguey, C., Chen, Z., & van der Ent, A. (2026). Monochromatic X-ray fluorescence spectroscopy for major and trace element analysis in plant science applications. *Plant and Soil*, *518*(1), 105–120. https://doi.org/10.1007/s11104-025-07984-5