

## Research paper

# Effects of elevation on growth, photosynthetic and Ni-accumulation responses in *Bornmuellera emarginata* (Brassicaceae)

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## ABSTRACT

*Bornmuellera emarginata* is a Balkan Ni-hyperaccumulator indicated as a promising candidate for agromining practices. Here, two elevation-contrasting accessions (250 m, Low Elevation plants LE, and at 1600 m, High Elevation plants, HE) were compared in terms of growth, photosynthetic activity, and Ni accumulation in controlled conditions to assess possible differences exploitable in practical applications. After two months of pot cultivation on garden and serpentine soil, plant biomass, gas exchanges and Ni concentrations were evaluated. A hydroponic trial with increasing NiSO<sub>4</sub> concentration was performed to evaluate Ni tolerance and accumulation.

Plants grown on serpentine soil showed reduced biomass as compared to those from garden soil, without any difference between LE and HE. In both accessions, growth in serpentine soil resulted in photochemical/biochemical limitations and reduction in stomatal conductance, especially in LE. HE showed low stomatal conductance in all the conditions, despite a greater stomatal density. Shoot Ni accumulation was higher in HE (~1600 µg Ni g<sup>-1</sup> d.w. and 1200 µg Ni g<sup>-1</sup> d.w. in HE and LE respectively), thus resulting in a significantly higher metal content per plant in respect to LE (~120 and ~85 µg plant<sup>-1</sup> in HE and LE respectively). In hydroponics, HE possessed higher Ni tolerance and accumulation in respect to LE.

In conclusion underlying the higher shoot metal amount of HE, Ni tolerance seemed to play a pivotal role, overtaking possible negative effects of reduced transpiration rates compared to LE. Our results point to the opportunity of exploring and exploiting the variation in accumulation level among *B. emarginata* accessions for the implementation of environmental restoration and metal cropping practices.

## 1. Introduction

Hyperaccumulators are plants with shoot concentrations of particular metals or metalloids reaching levels hundreds to thousands of times greater than is normal for most plants (van der Ent et al., 2013). They can take up and detoxify exceptional concentrations of elements, as an adaptation mechanism to metal rich soils, through adaptation of the metal regulation mechanisms shared by all higher plants (Clemens, 2017).

Nickel was the first element discovered to be hyperaccumulated in the ashes of the Central Italy-endemic plant *Odontarrhena bertolonii* (Minguzzi and Vergnano, 1948), and the latest one recognized as a plant micronutrient (Dixon et al., 1975). The physiological requirements for Ni are however extremely low compared to the other micronutrients (around 0.1 µg Ni g<sup>-1</sup> d.w. or lower, Gerendás, 1999), while the hyperaccumulation threshold is among the highest (1000 µg Ni g<sup>-1</sup> d.w.,

van der Ent et al., 2013). With 532 angiosperm species in 53 families (of which around 25% are Brassicaceae) and 130 genera worldwide, Ni is by far the most frequently accumulated metal (Reeves et al., 2018). Uptake through members of the ZRT/IRT-like (ZIP) family, as well as chelation with histidine and nicotianamine and compartmentalization through the tonoplast-localized transporters of the IREG/Ferroportin family (van der Pas and Ingle, 2019), are involved in Ni hyperaccumulation.

The discovery of hyperaccumulator plants has not only attracted the scientific interest in the study of their peculiar mechanisms of metal tolerance and accumulation, but it has also been a catalyst for the use of phytoextraction for remediating contaminated soils (Chaney et al., 1997). More recently, such application has given birth to the practice of agromining, in which hyperaccumulators are employed as ‘metal crops’ to sequester elements in harvestable biomass that can then be used to produce elemental metals (Kidd et al., 2018).

Nickel hyperaccumulators are often obligate endemics to ultramafic

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outcrops (mostly serpentine soils), and more rarely they grow either on or outside these substrates, including both accumulator and non-accumulator populations (Pollard et al., 2014). The infertility of serpentine soils is mainly due to phytotoxic levels of trace elements like Ni, Cr and Co, low levels of macronutrients and an unfavorable Ca:Mg ratio (Gonnelli and Renella, 2012). In Europe, the Balkans host the largest ultramafic outcrops of the continent, from Serbia and Bosnia at the North to Greece at the South, and represent an important hot-spot of biodiversity of Ni hyperaccumulators (Cecchi et al., 2018, 2010; Coppi et al., 2020). Among the Balkan hyperaccumulating plants, *Bormuelleria emarginata* (Boiss.) Resëtnik (synonyms: *Leptoplax emarginata* (Boiss.) O. E. Schulz, *Peltaria emarginata* (Boiss.) Hausskn.) (Resetnik et al., 2014) is one of the most remarkable. This plant is endemic to ultramafic soils in Greece, with a discontinuous distribution from the Pindus mountains to the island of Euboea. Unlike most other Ni-hyperaccumulators, it displays a wide altitudinal range from 250 to 1800, rarely 2100 m a.s.l. (Hartvig, 2002). It can accumulate up to 34,400  $\mu\text{g g}^{-1}$  foliar Ni (Reeves et al., 1980) and has been thus showed to have great potential for use in Ni-agromining systems, especially because of its high biomass production and ability to colonize disturbed grounds (Chardot et al., 2005; Lucisine et al., 2014; Pardo et al., 2018; Van Der Ent et al., 2015; Zhang et al., 2014). Regarding the hyperaccumulation mechanisms in *B. emarginata*, a higher apoplasmic Ni uptake has been evidenced in comparison to non-accumulating plants (Redjala et al., 2010), and citrate was found as the predominant ligand for Ni in stems while malate was predominant in leaves (Montargès-Pelletier et al., 2008). The occurrence of active Ni transport and a high root permeability to the metal have been reported (Bartoli et al., 2012; Coinchelin et al., 2012), as well as the preferential allocation of the metal in the leaf epidermis with the exclusion of the guard cells (Psaras et al., 2000). Besides these key results, *B. emarginata* can offer an ideal model system to investigate the relationship between ecophysiological traits and serpentine tolerance/Ni hyperaccumulation. One of the most striking features of the relatively restricted distribution of *B. emarginata* is the presence of accessions populating serpentine outcrops at very different elevation, such as in the Pindo Region, where the species can be found from the sea level to mountain elevations (Hartvig, 2002). By comparing the performance of accessions from two sites with contrasting elevations, this work aims to: i) assess possible differences in their growth and accumulation response when cultivated in controlled conditions on the same serpentine substrate, ii) assess whether different performance can be related to a different gas exchange and photosynthetic behavior on serpentine soils, iii) investigate if the two elevation-contrasting ecotypes have a different Ni tolerance and accumulation in hydroponic conditions. The transpiration stream is fundamental to deliver Ni to the final accumulation sink in hyperaccumulator shoots (Kachenko et al., 2008; Mesjasz-Przybyłowicz, 2001; Mesjasz-Przybyłowicz and Przybyłowicz, 2011), including *B. emarginata* (Bartoli et al., 2018). We therefore hypothesized that two accessions living at two contrasting elevations could have a different stomatal regulation generating a different metal accumulation in leaves when growing on the same serpentine soil in controlled conditions. The obtained results not only give new insights into the ecophysiology of Ni hyperaccumulation in *B. emarginata*, but also clarify whether it is worth to look for the best extractor at higher or lower elevations, since identifying the accessions with different tolerance and accumulation potential is a primary step for both a better understanding of the mechanisms and the success of practical applications.

## 2. Materials and methods

### 2.1. Plant material and soil sample collection

Plant material and seeds were collected from native accessions in July 2022 in the two contrasting Greek serpentine outcrops, one near the village of Kedros, at ca. 250 m (Low Elevation plants, LE) and one near

**Table 1**

Collection sites of *B. emarginata*, with accession, code, coordinates, elevation and Ni concentrations in the native soil ( $\mu\text{g g}^{-1}$  d.w., mean of 5 values  $\pm$  standard deviation, total concentration) and in roots and shoots of field collected plants ( $\mu\text{g g}^{-1}$  d.w., mean of 5 values  $\pm$  standard deviation).

Accession	Code	Lat, Long	Elevation (m)	[Ni] soil	[Ni] root	[Ni] shoot
Kedros	LE	39°10.028'	270	1960	3176	13,332
		22°02.587'		$\pm 39$	$\pm 391$	$\pm 949$
Katara Pass	HE	39°47.715'	1610	1876	8770	19,707
		21°12.132'		$\pm 62$	$\pm$	$\pm 2622$
					1462	

Katara Pass at ca. 1600 m a.s.l. (High Elevation plants, HE). Information about the accession localities is given in Table 1, with details on Ni concentrations in soil and plant samples. Ten bulk soil samples (ca. 20 g) were randomly collected at ca. 5–15 cm depth in the sampling area. These samples were then pooled together to obtain a single bulk soil sample of ca. 200 g. Bulk soil samples were dried at room temperature, sieved with a 2 mm mesh stainless steel sieve, and then oven-dried at 50 °C for 7 days (Pignattelli et al., 2012). From each sample, five sub-samples (0.5 g d.w.) were digested using 10 mL of 69% HNO<sub>3</sub> in a microwave digestion system (Mars 6, CEM) as in Bettarini et al. (2019). Nickel concentrations in the digests were determined by flame atomic absorption spectroscopy (AAS) using a PinAAcle 500 (Perkin Elmer, Waltham, MA, USA) and used to calculate metal concentration in soils. At the two sites, roots and shoots from five adult plants were randomly collected, together with over 1000 mature seeds from 10–15 fruiting plants at a distance of at least 10 m from each other. Harvested samples were carefully washed three times with deionized water, dried at 50 °C for 48 h (Selvi et al., 2017), and then the dried materials (0.1 g d.w.) analyzed as described for soils. Each plant analysis was replicated three times.

### 2.2. Pot cultivation

To compare serpentine tolerance and Ni-accumulation capacity in the two *B. emarginata* accessions, plants were cultivated in pots filled both with natural serpentine soil (Tuscany, Grosseto province), with a Ni total concentration of 1869  $\pm 66$  ( $\mu\text{g g}^{-1}$  d.w., mean of 5 samples  $\pm$  SD, pH 6.9–7.1. Average values of elements: 1.1  $\pm$  0.2, 5.0  $\pm$  0.1, 118.4  $\pm$  2.9, 91.8  $\pm$  7.6 mg g<sup>-1</sup> d.w. for K, Ca, Mg and Fe, and 1625  $\pm$  109, 2348  $\pm$  99, Cr 156  $\pm$  4  $\mu\text{g g}^{-1}$  d.w. for Mn, Co and Cr), and with garden soil (hereafter indicated as control soil. It was a commercial peat-clayed soil: Geotec Fioriture Tonerde, pH 5.5–6.0. Average values of elements: 4.7  $\pm$  0.5, 16.7  $\pm$  1.1 and 2.4  $\pm$  0.9 mg g<sup>-1</sup> d.w. for K, Ca and Mg, and 198.3  $\pm$  10.8 and 71.22  $\pm$  3.1  $\mu\text{g g}^{-1}$  d.w. for Fe, Mn, respectively. Nickel, Co and Cr values were below the detection limit). After seed sowing, pots with one plant each (12 pots/plants per treatment) were placed in a growth chamber (24/16 °C day/night; light intensity 300  $\mu\text{mol m}^{-2}\text{s}^{-1}$  in the PAR range, 16-h (day) photoperiod; relative humidity 60–65%). Plants were watered regularly twice a week and 12 individuals for treatment were sampled after two months to measure leaf area, as described in Colzi et al. (2022), and shoot fresh and dry weight. Subsequently, plants were rinsed with milliQ-water and roots were carefully washed with 10 mM Pb(NO<sub>3</sub>)<sub>2</sub> at 4 °C for 30 min to desorb metals adhering to the root cell wall, as in Bazihizina et al. (2015). Shoot fresh and dry weights were determined and the percentage of water content calculated as ((F<sub>W</sub>-D<sub>W</sub>)/F<sub>W</sub>)\* 100. Samples were analyzed for measuring Ni concentration as above described for soils. Ni content per plant shoot was calculated by multiplying the shoot dry biomass by the shoot Ni concentration.

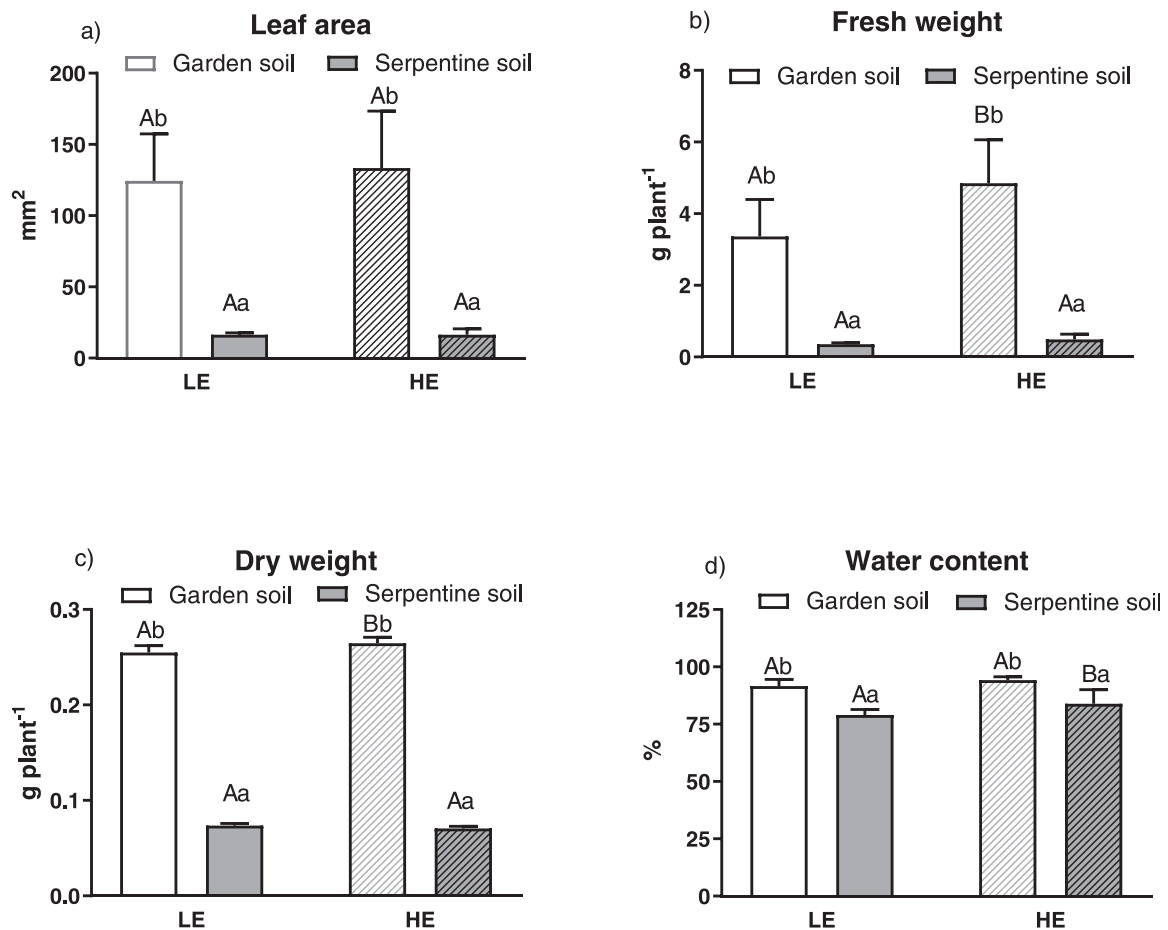


Fig. 1. Biometric traits at harvest of the two *B. emarginata* accessions grown for two months in pots with garden soil and with the same natural serpentine soil: (a) leaf area, (b) shoot fresh weight, (c) shoot dry weight, (d) shoot water content. Letters indicate significant differences among means (Tukey test), capital case among accessions and lower case within accessions. Values are means of 12 replicates  $\pm$  standard deviation. LE, low elevation plants; HE, high elevation plants.

### 2.3. Photosynthesis limitation analysis and chlorophyll fluorescence measurements

The portable photosynthesis system Li-6400 XT (LiCor Inc., Lincoln, NE, USA) was used to measure leaf gas exchange and chlorophyll a fluorescence parameters as in Bazihizina et al. (2015). Light-adapted chlorophyll fluorescence and gas exchanges were measured simultaneously using an open gas exchange system (Li-6400-40 leaf chamber fluorometer; Li-Cor, Lincoln, NE, USA) on the youngest fully expanded leaves at the end of the cultivation period. Measurements of net photosynthetic rate ( $A_n$ ) and stomatal conductance ( $g_s$ ) were determined with reference  $CO_2$  of  $400 \mu mol mol^{-1}$ , ambient relative humidity, flow rate of  $500 \mu mol s^{-1}$ , chamber temperature at  $20^\circ C$  and  $300 \mu mol m^{-2} s^{-1}$  of photosynthetically active radiation. To complete the chlorophyll fluorescence measurements, leaves were dark-adapted for 20 min and then a weak measuring beam was turned on to measure  $F_0$  prior to exposing the leaf to a single-pulse saturating flash of  $\sim 9.000 \mu mol m^{-2} s^{-1}$  intensity and 1 s duration to measure  $F_m$ . The following parameters were calculated (Maxwell and Johnson, 2000; Posch and Bennett, 2009): the maximum PSII efficiency ( $F_v/F_m$ ) measured in dark-adapted leaves; the effective PSII quantum yield ( $\Phi_{PSII}$ ) used to estimate the fraction of absorbed light used in photochemistry; the maximum PSII efficiency in the light ( $F_v'/F_m'$ ); photochemical quenching ( $qP$ ) used to estimate changes in the closure of reaction centers and to calculate the relative reduction state of the primary quinone receptor of PSII ( $1 - qP$ ); the nonphotochemical quenching ( $qN$  and  $NPQ$ ); and the electron transport rate (ETR).

$CO_2$  response curves ( $A_n/C_i$ ) were determined for all treatments with

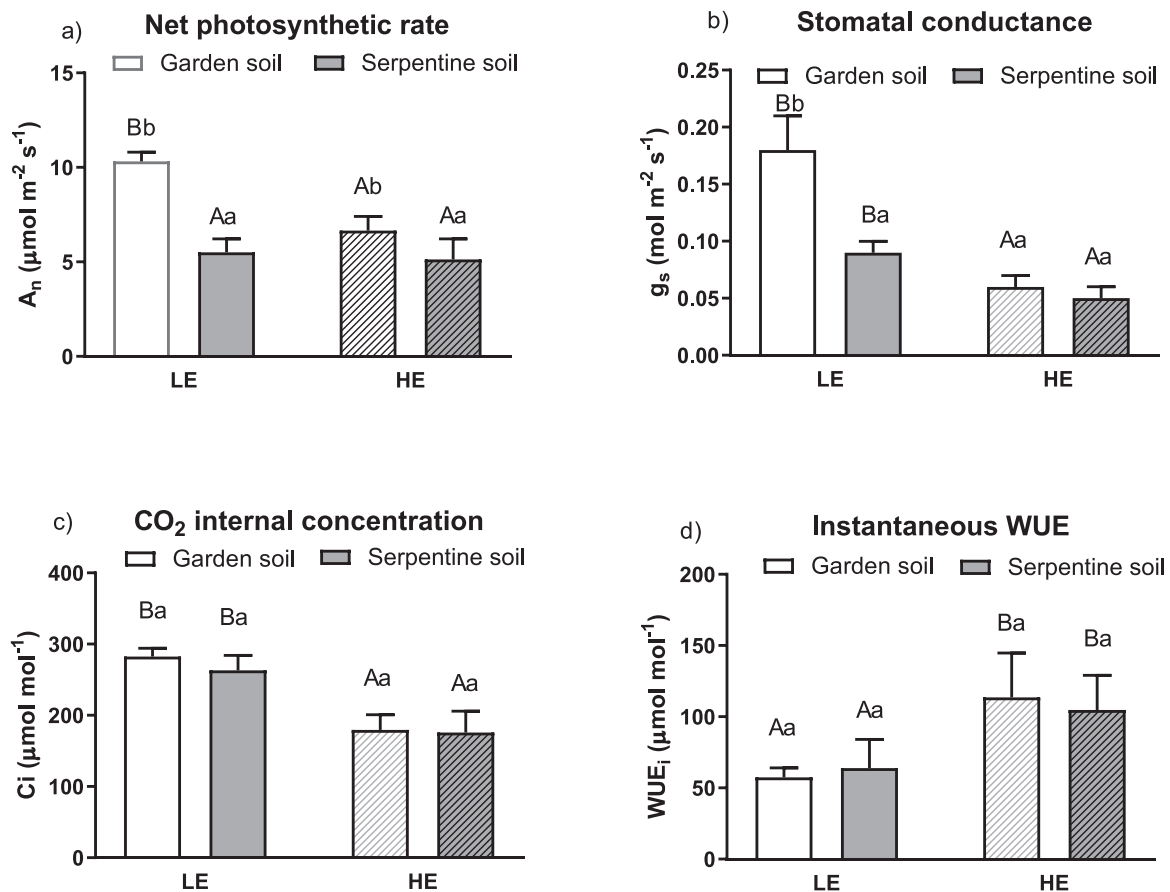
the following external  $CO_2$  concentrations: 400, 50, 100, 150, 250, 350, 500, 700, 900, 1200, and  $400 \mu mol mol^{-1}$ .  $A_n/C_i$  curves were analysed using the excel solver tool by Sharkey (2016) and the following key biochemical parameters were determined:  $V_{max}$  (maximum rate of carboxylation) and  $J_{max}$  (light-saturated rate of electron transport). Estimated  $V_{max}$  and  $J_{max}$  values were adjusted to  $25^\circ C$  in the Sharkey tool (named  $V_{max_{25}}$  and  $J_{max_{25}}$ , respectively). Stomatal limitations to photosynthesis ( $L_s$ ), calculated as the decrease in net  $CO_2$  assimilation attributable to stomata, was calculated as in Farquhar and Sharkey (1982).

### 2.4. Pigment concentration

To determine leaf pigment concentrations, young fully expanded leaves were harvested and snap-frozen in liquid  $N_2$ , stored at  $-80^\circ C$  and then freeze-dried. Cold 100% methanol was added to ground freeze-dried leaf tissues to determine pigment concentrations and subsequently samples were incubated in darkness at  $4^\circ C$  for 30 min. Following the incubation, samples were centrifuged at  $9300 g$  for 10 min at  $4^\circ C$ , and then the supernatant was removed, and the absorbance determined at 470, 665.2, and 652.4 nm, using a Tecan Infinite 200 Spectrophotometer (Männedorf, Switzerland). Chlorophyll a (Chla) and b (Chlb), and total carotenoid concentrations were obtained using the equations from Wellburn (1994).

### 2.5. Stomatal density and stomatal size

Stomatal density was assessed with a Zeiss Axio Observer Z1



**Fig. 2.** Net photosynthetic rate ( $A_n$ , a) and stomatal conductance ( $g_s$ , b), CO<sub>2</sub> internal concentration ( $C_i$ , c) and instantaneous water use efficiency (WUE<sub>i</sub>, d) at harvest of the two *B. emarginata* accessions grown for two months in pots with garden soil and with the same natural serpentine soil. Letters indicate significant differences among means (Tukey test), capital case among accessions and lower case within accessions. Values are means of 12 replicates  $\pm$  standard deviation. LE, low elevation plants; HE, high elevation plants.

fluorescence microscope (Carl Zeiss, Thornwood, NY). Young fully expanded leaves were collected at the end of the experiment and five areas of 0.155 mm<sup>2</sup> of the same leaf were observed and the stomata counted. Measurements were performed on three different leaves of the same plant. Stomatal density and size were measured manually using the Adobe Photoshop 7.0 software. Stomatal size was calculated according to (Ouyang et al., 2017) and 12 stomata were measured per leaf.

## 2.6. Nickel tolerance and accumulation in hydroponics

Seeds were sown in peat soil and 4-week-old seedlings were then transferred to hydroponic cultures in 1-L polyethylene pots (one plant per pot) containing a modified half-strength Hoagland's solution (Hoagland and Arnon, 1950) in milliQ-water (Millipore, Billerica, MA, USA) buffered with 2 mM 2-morpholinoethanesulphonic acid, adjusted to pH 5.5 with KOH. Background Ni concentration in the growing medium was about 0.1  $\mu\text{M}$  and this was considered as the control condition, as in (Bettarini et al., 2020). Nutrient solutions were changed every week, and plants were grown in the same growth chamber described above. After 3 weeks, plants of homogeneous size were selected and treated as in (Bettarini et al., 2021): root length of each plant was measured and subsequently plants were exposed for 7 days to a series of NiSO<sub>4</sub> concentrations (0, 50, 100, 250, 500, 1000, 2000, 3000  $\mu\text{M}$ , 12 plants per treatment), in a background solution of the same composition as the pre-culture solution. The applied Ni concentrations were previously proved to be adequate for serpentine hyperaccumulators (Bettarini et al., 2021, 2020) and were in the range of toxicity for plants not adapted to metal excess (5  $\mu\text{M}$ , Marschner, 1995). After seven days of

growth, root length of all the plants was measured again to assess the increment (calculated subtracting length values at the beginning of the treatment to values at the end of the treatment). Plants were rinsed with milliQ-water and roots were carefully desorbed as described above. Shoots and roots were dried at 70 °C for 24 h and Ni accumulation was determined as previously stated.

## 2.7. Data analyses

The experimental data points were fitted to the Brain-Cousens model for the analysis of the growth response to Ni treatment, as in Bettarini et al., (2021, 2020). Root length increment was measured as response variable for all tested accessions, while the Ni concentration of the growth medium was considered as the predictor variable. The Brain-Cousens model allows to validate the presence of significant hormetic effect and to estimate the following parameters: the external maximum stimulation dose (MSD), the maximum mean response (MAX, necessary for a reliable calculation of the percentage of the hormetic effect as  $[100 * (\text{MAX} - \text{length increment in control condition}) / \text{length increment in control condition}]$ , here named hormetic percentage = HP) and the half-maximal effective external concentration (EC50). The drc package (Ritz et al., 2015) as implemented in R Studio version R 3.4.3 (R Core Team 2017) was used to fit the curve of concentration-response data.

The differences among the means were analyzed by one-way and two-way ANOVA followed by HSD-Tukey test for post-hoc comparisons using GraphPad Prism 7 (GraphPad Software, San Diego, CA), after checking data normality distribution (assessed with the Shapiro-Wilk



**Table 2**

Photosynthetic biochemical parameters at harvest of the two *B. emarginata* accessions grown for two months in pots with garden soil and with the same natural serpentine soil. Letters indicate significant differences among means (Tukey test), capital case among accessions and lower case within accessions. Values are means of 12 replicates  $\pm$  standard deviation.  $V_{cmax25}$ , maximum rate of carboxylation adjusted at 25 °C;  $J_{max25}$ , light-saturated rate of electron transport adjusted at 25 °C; Ls, stomatal limitation to photosynthesis; Fv/Fm, maximum PSII efficiency in dark-adapted leaves; PhiPSII, effective PSII quantum yield; Fv'/Fm', maximum PSII efficiency in the light; qP, photochemical quenching used to estimate changes in the closure of reaction centers and to calculate the relative reduction state of the primary quinone receptor of PSII (1-qP); qN and NPQ, nonphotochemical quenching; ETR, electron transport rate. LE, low elevation plants; HE, high elevation plants.

Parameter	LE Garden soil	LE Serpentine soil	HE Garden soil	HE Serpentine soil
$V_{cmax25}$	62.17 $\pm$ 5.28 Ab	38.83 $\pm$ 3.65 Aa	87.33 $\pm$ 5.68 Bb	51.16 $\pm$ 7.29 Ba
$J_{max25}$	74.67 $\pm$ 2.20 Ab	46.17 $\pm$ 2.27 Aa	103.50 $\pm$ 4.33 Bb	66.17 $\pm$ 8.95 Ba
Ls	15.03 $\pm$ 2.49 Aa	31.89 $\pm$ 6.63 Ab	60.36 $\pm$ 4.78 Ba	53.85 $\pm$ 9.06 Aa
Fv/Fm	0.80 $\pm$ 0.01 Ab	0.75 $\pm$ 0.02 Aa	0.81 $\pm$ 0.01Aa	0.81 $\pm$ 0.03 Ba
Fv'/Fm'	0.46 $\pm$ 0.01 Ba	0.46 $\pm$ 0.01 Ba	0.44 $\pm$ 0.01 Ab	0.40 $\pm$ 0.02 Aa
PhiPSII	0.14 $\pm$ 0.01 Ab	0.09 $\pm$ 0.01 Aa	0.17 $\pm$ 0.01 Ab	0.11 $\pm$ 0.02 Aa
1-qP	0.70 $\pm$ 0.02 Ab	0.80 $\pm$ 0.01 Aa	0.61 $\pm$ 0.01 Bb	0.72 $\pm$ 0.03 Ba
qN	0.78 $\pm$ 0.05 Aa	0.79 $\pm$ 0.04 Aa	0.83 $\pm$ 0.02 Ba	0.90 $\pm$ 0.01 Bb
NPQ	1.90 $\pm$ 0.27 Aa	2.03 $\pm$ 0.41 Aa	2.23 $\pm$ 0.23 Aa	3.63 $\pm$ 0.31 Bb
ETR	79.64 $\pm$ 3.58 Ab	52.70 $\pm$ 1.74 Aa	98.68 $\pm$ 3.31 Bb	64.36 $\pm$ 9.37 Ba

test).

### 3. Results

#### 3.1. Plant growth on serpentine soil

In both accessions, plants grown on serpentine soils displayed a significantly lower leaf area ( $\sim 20 \text{ mm}^2$ ) compared to those grown on garden soil ( $\sim 125 \text{ mm}^2$ ), with a reduction of 86.8% and 87.7% for LE and HE respectively (Fig. 1a), without any significant interaction accession\*treatment (Table 1Sa). A similar serpentine-imposed decrease was present in shoot fresh and dry weight (89.4% and 89.9% in fresh weight, 71.2% and 73.5% in dry weight for LE and HE respectively, Fig. 1b and c), and for this parameter there was a significant interaction accession\*treatment, with HE plants showing a higher mass than LE when grown on garden soil (Table S2). The percentage of shoot water content was significantly higher in plants from garden soil as compared with samples from serpentine soil (reduction of 14.0% and 10.9% in LE and HE respectively, Fig. 1d) with HE showing a higher value than LE, but without significant accession\*treatment interaction (Table S1d).

#### 3.2. Gas exchange parameters on serpentine soil

For both accessions there was a significant decrease in net photosynthetic rate ( $A_n$ ) when plants were grown on serpentine soil ( $\sim 5 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) compared to those in control soil ( $\sim 10$  and  $\sim 6.5 \mu\text{mol m}^{-2}\text{s}^{-1}$  for a 46.6% and 22.9% decrease in LE and HE respectively, Fig. 2a). On garden soil, the LE accession had a higher  $A_n$  than HE, with a significant interaction accession\*treatment (Table S2a). Regarding stomatal conductance rates ( $g_s$ ), a significant serpentine-induced decrease (around 50%) was present only in LE (from  $\sim 0.175 \text{ mol m}^{-2}\text{s}^{-1}$  to  $\sim 0.08 \text{ mol m}^{-2}\text{s}^{-1}$ , Fig. 2b) that also showed

values always higher than HE ( $0.06 \text{ mol m}^{-2}\text{s}^{-1}$  and  $0.05 \text{ mol m}^{-2}\text{s}^{-1}$  on garden and serpentine soil respectively, Fig. 2b) with significant accession\*treatment interaction (Table S2b). In both accessions, the internal concentration of  $\text{CO}_2$  and the instantaneous water use efficiency ( $C_i$  and  $\text{WUE}_i$ , Fig. 2c and d) did not change upon serpentine exposure, with  $C_i$  higher in LE and  $\text{WUE}_i$  in HE, without a significant accession\*treatment interaction (Table S2 c and d).

The key parameters, calculated from the  $A_n/C_i$  curves (Fig. S1) and the chlorophyll fluorescence measurements, are reported in Table 2. The values of  $V_{cmax25}$ ,  $J_{max25}$ , PhiPSII, and ETR were significantly lower in plants from serpentine soil in respect to those from control soil in both accessions. On the other hand, the maximum PSII efficiency (Fv/Fm) decreased only in LE while the maximum light use efficiency of light acclimated PSII centers (Fv'/Fm') decreased only in HE. For both accessions, the excitation pressure (1-qP), a measure of PSII vulnerability to photoinactivation, increased in serpentine soils, but it always remained lower in HE. The serpentine soil induced an increase in non-photochemical quenching (qN and NPQ) in HE but not in LE. The interaction accession\*treatment was significant for all the parameters except PhiPS2 and qP (Table S3). Substantial differences were also observed for calculated stomatal limitations to photosynthesis in the two accessions. Indeed, while in LE stomatal limitation was low under control conditions and increased only in serpentine soils, HE plants had inherently high stomatal limitations independently of the soil type.

#### 3.3. Stomatal density and size on serpentine soil

Regarding stomata, plants from serpentine soils showed a lower stomatal density compared to those grown on control soil for both the lower and the upper surface (Fig. 3a and b). The percentage of reduction was higher in LE (around 28% and 18% for LE and HE respectively) in the lower surface and similar in the upper one (18% and 21% for LE and HE respectively). HE displayed a higher stomatal density in all the treatments in comparison to LE, with a significant interaction accession\*treatment in the case of the upper surface (Table S4). The stomatal size varied only in HE (Fig. 3c and d), that when grown on serpentine soil showed a higher value in respect to plants from control soil, and to LE plants from serpentine soil for the lower surface, with an interaction accession\*treatment always significant (Table S4).

#### 3.4. Pigment concentration on serpentine soil

Besides the higher Chl a and Car concentrations in LE compared to HE when grown on control soil, all samples from serpentine soils displayed similar lower values of pigment concentrations (Fig. 4). The interactions treatment\*accessions were significant for Chl a and Car concentrations (Table S5).

#### 3.5. Nickel accumulation on serpentine soil

Nickel concentration in roots was negligible in plants from control soil, but significantly higher in those from serpentine soil with HE showing the highest value ( $\sim 260 \mu\text{g Ni g}^{-1} \text{ d.w.}$  vs  $\sim 190 \mu\text{g Ni g}^{-1} \text{ d.w.}$  in LE) and a significant interaction accession\*treatment (Fig. 5a, Table S6a). Nickel levels in shoots from plants grown on serpentine soil exceeded the  $1000 \mu\text{g Ni g}^{-1} \text{ d.w.}$  threshold and displayed a similar trend in roots, with HE always having significantly higher metal concentrations ( $\sim 1700 \mu\text{g Ni g}^{-1} \text{ d.w.}$  vs  $1200 \mu\text{g Ni g}^{-1} \text{ d.w.}$  in LE, Fig. 5b, Table S6b). For plants grown on serpentine substrate, the Ni translocation factor (shoot-to-root ratio of Ni concentration) was very similar, 6.33 and 6.53 for LE and HE respectively. Concerning shoot Ni content per plant (Fig. 5c), HE showed significantly higher values than LE on serpentine soil ( $\sim 120$  and  $\sim 85 \mu\text{g plant}^{-1}$  respectively), with a significant interaction accession\*treatment (Table S6c).

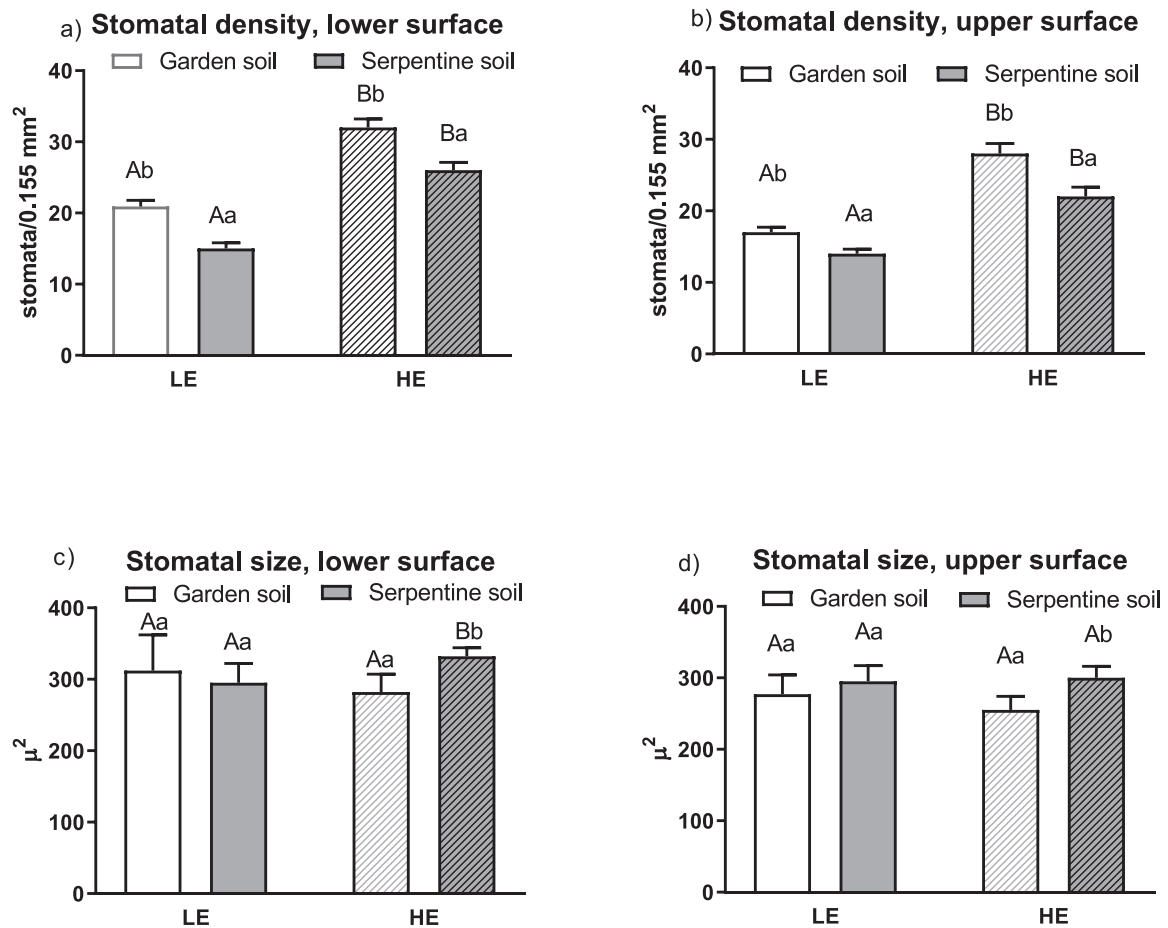


Fig. 3. Stomatal density and size at harvest of the two *B. emarginata* accessions grown for two months in pots with garden soil and with the same natural serpentine soil: a) Stomatal density of the leaf lower surface, b) Stomatal density of the leaf upper surface, c) Stomatal size of the leaf lower surface, d) Stomatal size of the leaf upper surface. Letters indicate significant differences among means (Tukey test), capital case among accessions and lower case within accessions. Values are means of 12 replicates  $\pm$  standard deviation. LE, low elevation plants; HE, high elevation plants.

### 3.6. Nickel tolerance and accumulation in hydroponics

Fig. 6 reports the root length variations of the two *B. emarginata* accessions in presence of increasing  $\text{NiSO}_4$  concentrations after 7 days of treatment (Fig. 6). A significantly higher growth increment compared to control conditions was present in the low-dose zone. LE displayed significant increments up to the treatment  $250 \mu\text{M NiSO}_4$  while HE up to  $500 \mu\text{M NiSO}_4$ . A general reduction in root length increment occurred at the highest metal levels, starting at  $500$  and  $1000 \mu\text{M NiSO}_4$  for LE and HE respectively. A significant inter-accession variation in root elongation in response to Ni treatments and a significant interaction accession\*treatment was scored (Table S7).

A significant data fitting was provided by the Brain-Cousens hormetic model, with the lack-of-fit test resulting in p-values higher than 0.05 (Table 3) and thus validating the model. In respect to LE, HE displayed lower MAX and HP values and higher MSD and EC50 values, with a significant difference in the latter.

In both accessions, Ni concentration in roots and shoots increased with increasing external Ni concentration (Fig. 7). In almost all treatments, shoots displayed Ni concentrations higher than roots, with the levels of accumulation always higher in HE. Significant variation between the accessions and a significant effect accession\*treatment were present for both roots and shoots (Table S8).

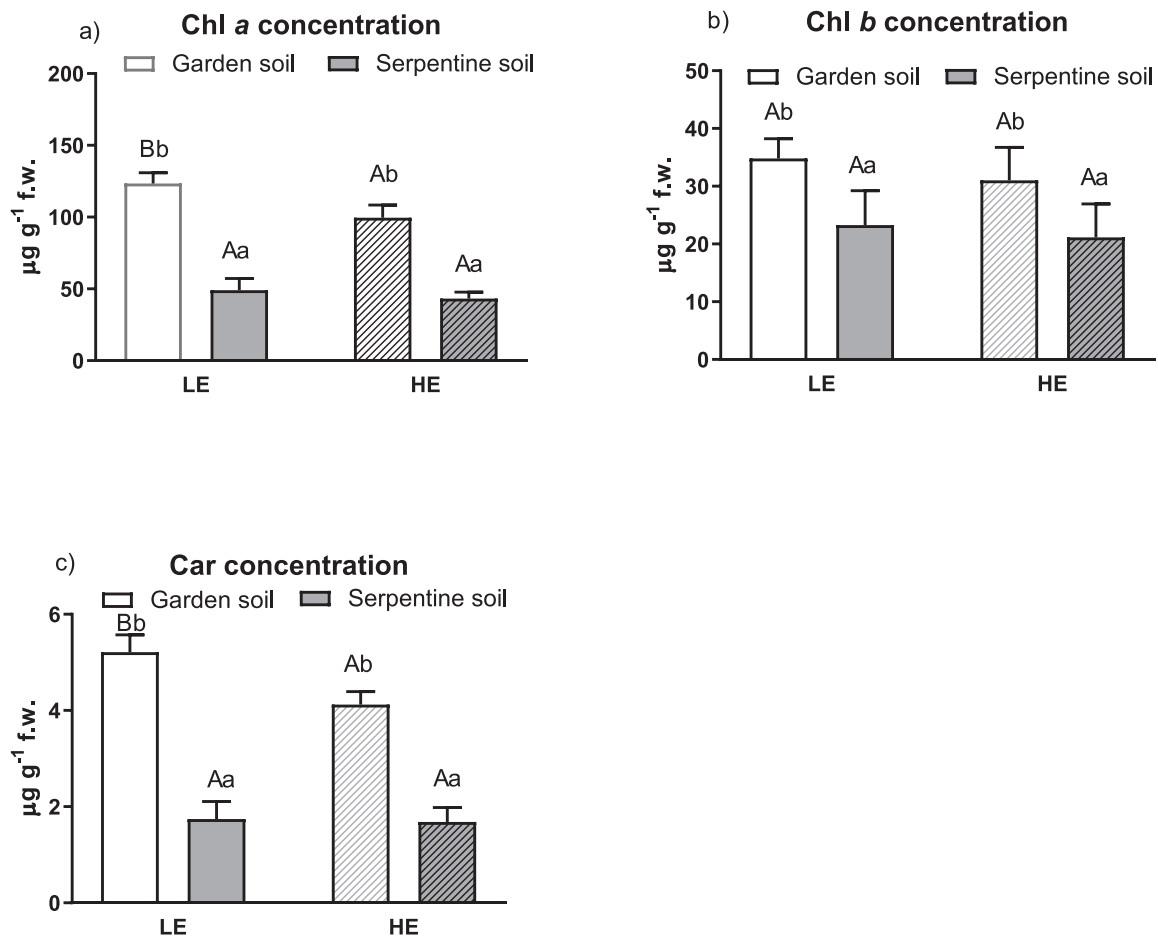
## 4. Discussion

### 4.1. Plant growth on serpentine soil

For both accessions, growth on serpentine soil led to reductions for all analysed biometrical traits compared to control plants grown on garden soil. This was likely due to the typical infertility of the ultramafic substrate (Kazakou et al., 2008). The general reduction of leaf area in serpentine-grown plants was in line with the decreased fresh and dry biomass production. Similar results were obtained by Colzi et al. (2023) in the Ni hyperaccumulator *O. bertolonii* and is considered as an adaptive mechanism to reduce transpiration on the usually open and dry serpentine soils (Coppi et al., 2022; Lazzaro et al., 2021). In any case, the two accessions showed a similar tolerance to the serpentine substrate in controlled conditions, as there was a similar growth reduction compared to garden soil plants. Interestingly, the accessions showed differences in their water content on serpentine soil, with HE displaying higher values and a lower serpentine-induced decrease in comparison to values on garden soil suggesting overall improved water relations in HE for both substrates.

### 4.2. Gas exchanges and photosynthetic parameters on serpentine soil

In garden soils the highland accession HE had significantly reduced net photosynthetic rate compared to the lowland accession LE. Decline in photosynthetic activities are generally ascribed to metabolic impairments or stomatal limitations (James et al., 2006). In our study, the low



**Fig. 4.** Pigment concentration at harvest of the two *B. emarginata* accessions grown for two months in pots with garden soil and with the same natural serpentine soil: a) Chlorophyll a concentration, b) Chlorophyll b concentration, c) Carotenoid concentration. Letters indicate significant differences among means (Tukey test), capital case among accessions and lower case within accessions. Values are means of 12 replicates  $\pm$  standard deviation. LE, low elevation plants; HE, high elevation plants.

photosynthetic rates in HE grown in garden soils were mainly due to stomatal limitations, as also confirmed by the lower  $C_i$  values compared to those measured in LE. The greater  $V_{cmax_{25}}$  and  $J_{max_{25}}$  values measured in HE further confirmed that there were no biochemical limitations on photosynthesis. Surprisingly, however, HE displayed lower stomatal conductance and greater stomatal limitations despite the fact this accession had a higher stomatal density (1.5-fold for the lower surface and 1.6-fold for the upper surface). The uncoupling between stomatal density and conductance (that resulted in increase in stomatal limitations) in HE suggests a decrease in plant hydraulic conductivity, or other adjustments linked to the characteristics of the site of origin, that resulted in limited stomatal opening, and led to the observed increase in intrinsic water use efficiency in both substrates. Indeed, the higher elevation location can represent a more challenging environment, due to a combination of multiple factors, from changes in  $\text{CO}_2$  partial pressure, temperatures (e.g., a few degree drop in temperature would likely lead to cold-induced increases in soil resistance in the water flow from the soil to the leaves, (Magnani and Borghetti, 1995), strong winds and high solar and UV radiations (Cotado and Munné-Bosch, 2020). For instance, substantial difference in atmospheric pressure can act as an evolutionary selective agent for different leaf functional traits and stomatal density and  $V_{cmax}$  have often been reported to increase with increasing elevation (Wang et al., 2017). These leaf functional traits alterations along an elevational gradient can be either environmentally and/or genetically controlled. The results from our study indicate that these differential responses in stomatal density among the two accessions were at least in part genetically controlled, as the differences remained

as both were grown in the same controlled conditions on garden soil at low elevation. Lower leaf pigment concentrations in HE under control conditions further support this hypothesis as the declines in leaf pigment amount is considered as an adaptive response to high elevation (Fatima et al., 2022), as reduced biomass investment in photosynthetic pigments would reduce oxidative damage by decreasing light absorption and preventing ROS production at high elevations.

In serpentine soils, the constitutively reduced stomatal opening and limitations to photosynthesis in HE enabled them to maintain conductance and photosynthetic rates comparable to control plants. On the contrary, in LE, there was a 44 to 52% drop in photosynthetic and stomatal conductance rates when grown on serpentine substrate. While significant biochemical limitation on photosynthesis emerged after 2-months growth in serpentine soils in both accessions, stomatal limitations doubled for LE while they remained similar to controls in HE. This would explain, at least in part, why HE maintained carbon acquisition rates only slightly lower in serpentine soil as compared to garden soil, notwithstanding the increases in biochemical limitations common to the two accessions. The higher capacity to dissipate excess excitation energy in HE plants provided another explanation. Indeed, thermal energy dissipation is the first line of defense against detrimental effects of excess excitation energy preventing the onset of photoinhibition (Posch and Bennett, 2009). Indeed, while in both accessions there was a decline in  $\Phi_{PSII}$  and ETR (i.e. in the fraction of light used for photochemistry and light-driven electron flow), only in HE there was a concomitant decline in  $F_v'/F_m'$  and an increase in thermal energy dissipation, as indicated by higher rates of non-photochemical fluorescence quenching ( $q_N$  and

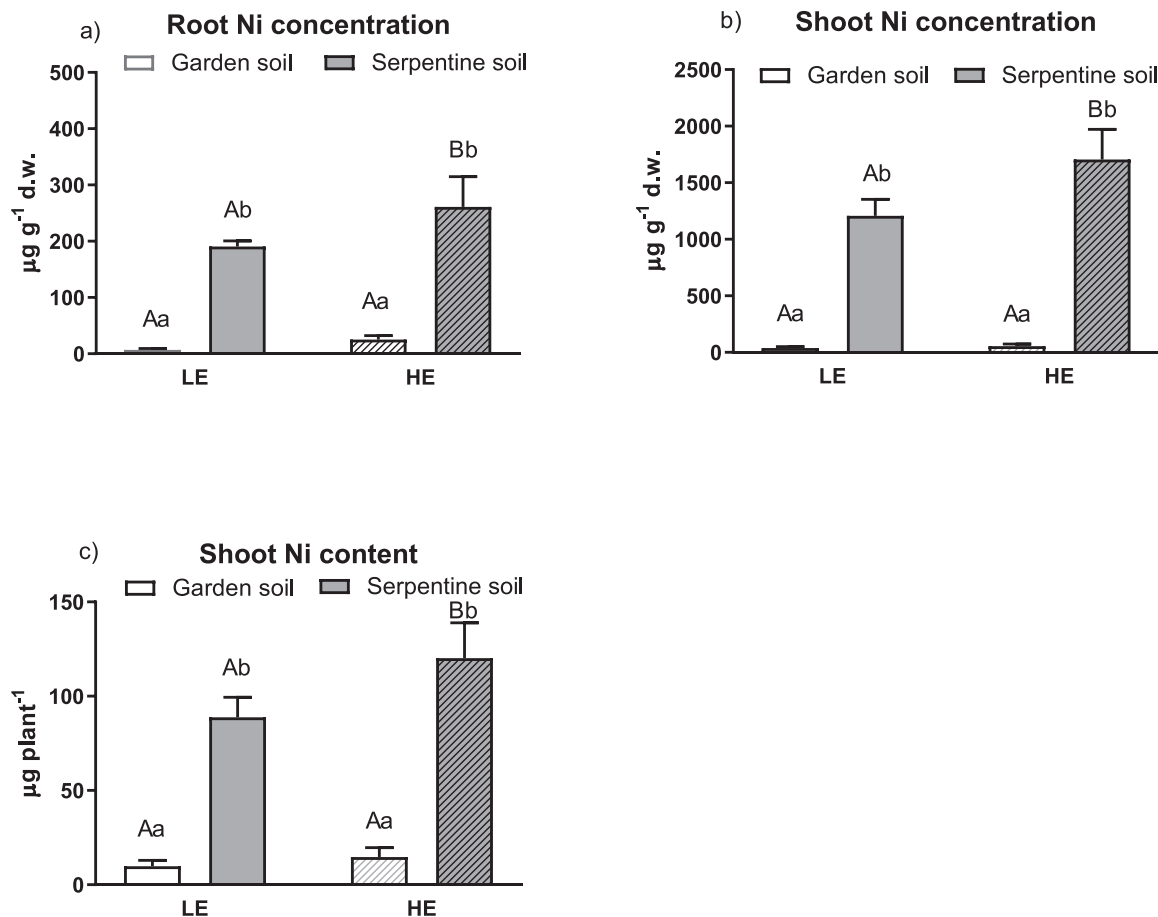


Fig. 5. Nickel concentration at harvest of the two *B. emarginata* accessions grown for two months in pots with garden soil and with the same natural serpentine soil: a) metal concentration in roots, b) metal concentration in shoots, c) metal content per plant shoots. Letters indicate significant differences among means (Tukey test), capital case among accessions and lower case within accessions. Values are means of 12 replicates  $\pm$  standard deviation. LE, low elevation plants; HE, high elevation plants.

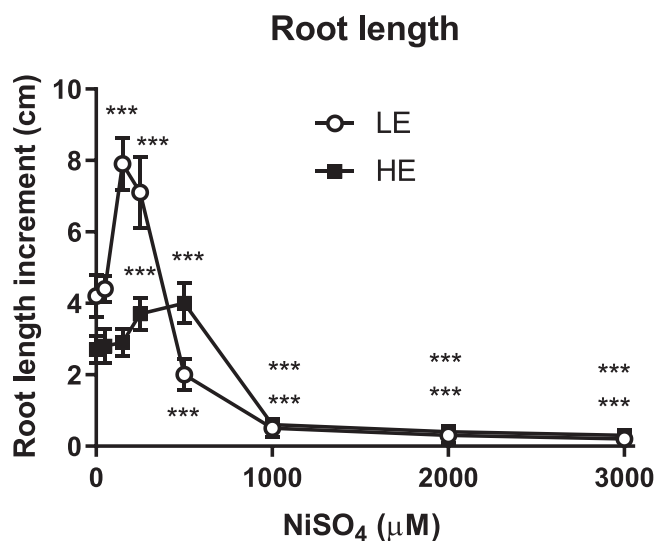


Fig. 6. : Increment in root length (cm) in the two accessions of *B. emarginata* treated with increasing  $\text{NiSO}_4$  concentrations. Asterisks indicate the significant differences in root length within each accessions according to the Tukey's test. \*\*\*  $p < 0.001$ . Values are means of 12 replicates  $\pm$  standard deviations. LE, low elevation plants; HE, high elevation plants.

Table 3

Parameters of the root dose-response curves: p-values of the lack-of-fit test and growth parameters calculated with the Brain-Cousens model for the two *B. emarginata* accessions grown at eight  $\text{NiSO}_4$  concentrations. Letters indicate significant differences among accessions according to the Tukey's test ( $p < 0.05$ ). LE, low elevation plants; HE, high elevation plants.

Accession	Lack-of-fit test	MSD ( $\mu\text{M}$ )	MAX (cm)	HP (%)	EC50 ( $\mu\text{M}$ )
LE	0.072	199	7.6	80.9	517 $\pm$ 95 a
HE	0.189	522	4.0	32.5	866 $\pm$ 117 b

NPQ). This energy dissipation appeared to be sufficient to prevent further negative effects and protect the photosynthetic machinery against serpentine-induced stress in light, as  $F_v/F_m$  values remained similar to control plants. However, this induction of photoprotective mechanisms did not occur in LE plants, and long-term exposure to serpentine soils led to a drop  $F_v/F_m$  and an increase in excitation pressure (1-qP), suggesting that the lack of energy dissipation led to permanent PSII damage. Interestingly, notwithstanding the improved photosynthetic capacity and performance in HE, this did not translate in differences in biomass production over the 2-month period, since the accessions appeared equally serpentine tolerant.

#### 4.3. Nickel accumulation on serpentine soil

Regarding plant metal concentration, as expected for Ni-hyperaccumulators, shoot Ni concentration on serpentine soil was



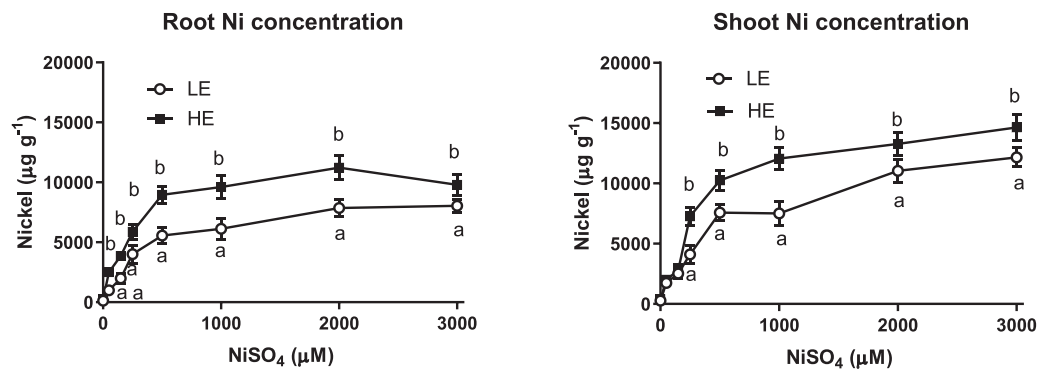


Fig. 7. Nickel accumulation ( $\mu\text{g g}^{-1}$  d.w.) in roots and shoots of the two *B. emarginata* accessions treated with eight  $\text{NiSO}_4$  concentrations for seven days. Values are means of 12 replicates  $\pm$  standard deviation. When present, letters indicate significant differences among accessions according to the Tukey's test ( $p < 0.05$ ) for each concentration. LE, low elevation plants; HE, high elevation plants.

invariably higher than root concentration and above the threshold of  $1000 \mu\text{g g}^{-1}$  d.w. (Deng et al., 2018; van der Ent et al., 2013). Nevertheless, there were significant differences between the two accessions, with HE showing higher Ni concentration than LE in both roots and shoots, regardless of the similar translocation factor. The metabolic cost of this higher accumulation capacity in HE could be one of the causes of the similar growth displayed by the two accessions on serpentine soil despite the more efficient photosynthetic performance of the former plants in respect to LE. Apparently, this higher metal accumulation was not related to a higher stomatal conductance and transpiration stream, since HE showed lower  $g_s$  and higher WUE. The transpiration-driven Ni transport has already been indicated as a concurring mechanism of metal hyperaccumulation in this species (Bartoli et al., 2018), but our results suggested that it was unlikely involved in generating differences in the level of accumulation between these two elevation-contrasting *B. emarginata* accessions. In addition, despite similar biomass on serpentine soil, the 1.4-fold higher Ni concentration in HE resulted in significantly greater total amount of Ni in the above-ground parts per plant compared to LE. High Ni concentration in shoots is one of the basic features for Ni agromining applications (Kidd et al., 2018) and resulted a fundamental discriminant between these two *B. emarginata* accessions since they showed the same tolerance and biomass production on serpentine soil. Interestingly, also the Ni concentration in the plants collected in the field were higher in HE, despite similar soil Ni concentrations in both sites of origin.

#### 4.4. Plant growth and Ni accumulation in hydroponics

In both accessions Ni altered root growth in a dose-dependent manner, with a stimulation at low Ni doses and a reduction above 500 and 1000  $\mu\text{M}$  for LE and HE respectively. These increases in root growth with low Ni concentrations were in line with recent results obtained for other Ni-hyperaccumulating taxa from the Balkan and Italian serpentine outcrops (Bettarini et al., 2021; Colzi et al., 2023). The stimulatory effect of low Ni concentrations on growth in hyperaccumulators has been partly attributed to an indirect ameliorative action of the metal on the plant photosynthetic machinery linked with a high metal requirement (Scartazza et al., 2022) and a parallel hyperaccumulation-mediated Ni cytosolic depletion in the low-dose zone (Bettarini et al., 2021).

The two dose-response curves significantly fitted the Brain-Cousens model (BRAIN and COUSENS, 1989), and the magnitude of the stimulating effect (HP) was always well above the 10% hormetic threshold proposed by Calabrese and Blain (2009). The hormetic parameters showed differences between the accessions, significantly for EC50 with HE resulting more Ni tolerant. This higher EC50 value was coupled to a higher Ni concentration in the substrate required for the maximum stimulatory effect (MSD), as found for *Odontarrhena* accessions (Bettarini et al., 2021; Colzi et al., 2023). The metabolic cost of this higher

tolerance could be related to the lower root size (quantified by the lower MAX) of the HE accession in hydroponics, since metal adaptation is supposed to leave less energy for plant growth (Maestri et al., 2010).

Concerning Ni accumulation, metal shoot concentration in the two accessions was always higher than root concentration, as typical of hyperaccumulating plants (Deng et al., 2018). Remarkably, HE showed higher values of Ni accumulation in both roots and shoots. To reach their maximal growth, therefore, HE seemed not only to need higher external Ni concentrations, but also higher ones inside their tissues. As already suggested for some *Odontarrhena* accessions (Bettarini et al., 2021; Colzi et al., 2023), this effect was likely due to more efficient hyper-accumulation mechanisms inducing higher Ni compartmentation and, therefore, cytosolic depletion.

Despite the same Ni concentration of the substrates of origin and the same tolerance to the serpentine soil showed by pot cultivated plants, our hydroponic data demonstrated that tolerance to Ni alone can be different and, probably, this higher tolerance displayed by HE could be the factor that determined the higher accumulation levels in the field collected samples and in the experiments in controlled conditions, with tolerance as the main driving force for accumulation, as suggested for *Noccaea goesingensis* (Krämer et al., 1997). Furthermore, a higher Ni tolerance could also be one of the causes of the lower serpentine-imposed decrease in photosynthetic performance in HE and its costs would then explain, at least in part, the lack of effect in biomass production mentioned above.

## 5. Conclusions

This study provides insights into some inherent physiological differences between two accessions of the Ni-hyperaccumulator *Bornmuellera emarginata* from elevation-contrasting environments. In pot cultivation, as compared to lowland plants, highland ones showed improved photosynthetic capacity, reduced conductance rates, and increased intrinsic water use efficiency, as typical of plants from elevated habitats. Tolerance to serpentine soil in pot trials was similar in both the accessions, but highland plants showed better photosynthetic performance and higher Ni accumulation, probably due to increased metal tolerance as revealed by a hydroponic trial.

Therefore, our results demonstrated that the response to serpentine soil in controlled conditions can be different in the accessions of this serpentine-endemic hyperaccumulator, with different amount of shoot Ni concentrations. Thus, the expansion of the study to a higher number of accessions is encouraged for implementing both the knowledge of the hyperaccumulation mechanisms and the phytotechnologies aimed at the recovery of trace metals from ultramafic soils using this species.

## CRedit authorship contribution statement

**Bazihizina Nadia:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Colzi Ilaria:** Writing – review & editing, Investigation, Formal analysis. **Gonnelli Cristina:** Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization. **Bettarini Isabella:** Writing – review & editing, Investigation, Formal analysis. **Selvi Federico:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.envexpbot.2024.105652](https://doi.org/10.1016/j.envexpbot.2024.105652).

## References

- Bartoli, F., Coinchelin, D., Robin, C., Echevarria, G., 2012. Impact of active transport and transpiration on nickel and cadmium accumulation in the leaves of the Ni-hyperaccumulator *Leptoplax emarginata*: a biophysical approach. *Plant Soil*. <https://doi.org/10.1007/s11104-011-0885-9>.
- Bartoli, F., Royer, M., Coinchelin, D., Le Thiec, D., Rose, C., Robin, C., Echevarria, G., 2018. Multiscale and age-dependent leaf nickel in the Ni-hyperaccumulator *leptoplax emarginata*. *Ecol. Res.* <https://doi.org/10.1007/s11284-018-1594-0>.
- Bazihizina, N., Colzi, I., Giorni, E., Mancuso, S., Gonnelli, C., 2015. Photosynthesizing on metal excess: copper differently induced changes in various photosynthetic parameters in copper tolerant and sensitive *Silene paradoxa* L. populations. *Plant Sci.* <https://doi.org/10.1016/j.plantsci.2014.12.015>.
- Bettarini, I., Colzi, I., Coppi, A., Falsini, S., Echevarria, G., Pazzagli, L., Selvi, F., Gonnelli, C., 2019. Unravelling soil and plant metal relationships in Albanian nickel hyperaccumulators in the genus *Odontarrhena* (syn. *Alyssum* sect. *Odontarrhena*, Brassicaceae). *Plant Soil* 440. <https://doi.org/10.1007/s11104-019-04077-y>.
- Bettarini, I., Colzi, I., Gonnelli, C., Pazzagli, L., Reeves, R.D., Selvi, F., 2020. Inability to accumulate Ni in a genus of hyperaccumulators: the paradox of *Odontarrhena sibirica* (Brassicaceae). *Planta* 252. <https://doi.org/10.1007/s00425-020-03507-x>.
- Bettarini, I., Gonnelli, C., Selvi, F., Coppi, A., Pazzagli, L., Colzi, I., 2021. Diversity of Ni growth response and accumulation in Central-Eastern Mediterranean *Odontarrhena* (Brassicaceae) populations on and off serpentine sites. *Environ. Exp. Bot.* 186 <https://doi.org/10.1016/j.envexpbot.2021.104455>.
- BRAIN, P., COUSENS, R., 1989. An equation to describe dose responses where there is stimulation of growth at low doses. *Weed Res.* 29 <https://doi.org/10.1111/j.1365-3180.1989.tb00845.x>.
- Calabrese, E.J., Blain, R.B., 2009. Hormesis and plant biology. *Environ. Pollut.* <https://doi.org/10.1016/j.envpol.2008.07.028>.
- Cecchi, L., Gabbriellini, R., Arnetoli, M., Gonnelli, C., Hasko, A., Selvi, F., 2010. Evolutionary lineages of nickel hyperaccumulation and systematics in European allysaeae (Brassicaceae): evidence from nrDNA sequence data. *Ann. Bot.* <https://doi.org/10.1093/aob/mcq162>.
- Cecchi, L., Bettarini, I., Colzi, I., Coppi, A., Echevarria, G., Pazzagli, L., Bani, A., Gonnelli, C., Selvi, F., 2018. The genus *Odontarrhena* (Brassicaceae) in Albania: Taxonomy and nickel accumulation in a critical group of metallophytes from a major serpentine hot-spot. *Phytotaxa.* <https://doi.org/10.11646/phytotaxa.351.1.1>.
- Chaney, R.L., Malik, M., Li, Y.M., Brown, S.L., Brewer, E.P., Angle, J.S., Baker, A.J.M., 1997. Phytoremediation of soil metals. *Curr. Opin. Biotechnol.* [https://doi.org/10.1016/S0958-1669\(97\)80004-3](https://doi.org/10.1016/S0958-1669(97)80004-3).
- Chardot, V., Massoura, S.T., Echevarria, G., Reeves, R.D., Morel, J.L., 2005. Phytoextraction potential of the nickel hyperaccumulators *Leptoplax emarginata* and *Borrmuelleria tymphaea*. *Int. J. Phytoremediat.* <https://doi.org/10.1080/16226510500327186>.
- Clemens, S., 2017. How metal hyperaccumulating plants can advance Zn biofortification. *Plant Soil.* <https://doi.org/10.1007/s11104-016-2920-3>.
- Coinchelin, D., Bartoli, F., Robin, C., Echevarria, G., 2012. Ecophysiology of nickel phytoaccumulation: a simplified biophysical approach. *J. Exp. Bot.* <https://doi.org/10.1093/jxb/ers230>.
- Colzi, I., Renna, L., Bianchi, E., Castellani, M.B., Coppi, A., Pignattelli, S., Loppi, S., Gonnelli, C., 2022. Impact of microplastics on growth, photosynthesis and essential elements in *Cucurbita pepo* L. *J. Hazard. Mater.* <https://doi.org/10.1016/j.jhazmat.2021.127238>.
- Colzi, I., Gonnelli, C., Bettarini, I., Selvi, F., 2023. Polyploidy affects responses to Nickel in Ni-hyperaccumulating plants: evidence from the model species *Odontarrhena bertolonii* (Brassicaceae). *Environ. Exp. Bot.* <https://doi.org/10.1016/j.envexpbot.2023.105403>.
- Coppi, A., Baker, A.J.M., Bettarini, I., Colzi, I., Echevarria, G., Pazzagli, L., Gonnelli, C., Selvi, F., 2020. Population genetics of *odontarrhena* (Brassicaceae) from albania: the effects of anthropic habitat disturbance, soil, and altitude on a nihyperaccumulator plant group from a major serpentine hotspot. *Plants* 9. <https://doi.org/10.3390/plants9121686>.
- Coppi, A., Lazzaro, L., Selvi, F., 2022. Plant mortality on ultramafic soils after an extreme heat and drought event in the Mediterranean area. *Plant Soil.* <https://doi.org/10.1007/s11104-021-05179-2>.
- Cotado, A., Munné-Bosch, S., 2020. Plasticity in the growth habit prolongs survival at no physiological cost in a monocarpic perennial at high altitudes. *Ann. Bot.* <https://doi.org/10.1093/aob/mcz202>.
- Deng, T.H.B., van der Ent, A., Tang, Y.T., Sterckeman, T., Echevarria, G., Morel, J.L., Qiu, R.L., 2018. Nickel hyperaccumulation mechanisms: a review on the current state of knowledge. *Plant Soil.* <https://doi.org/10.1007/s11104-017-3539-8>.
- Dixon, N.E., Gazzola, C., Blakeley, R.L., Zerner, B., 1975. Jack Bean Urease (EC 3.5.1.5). A metalloenzyme. A Simple Biological Role for Nickel? *J. Am. Chem. Soc.* <https://doi.org/10.1021/ja00847a045>.
- Farquhar, G.D., Sharkey, T.D., 1982. Stomatal conductance and photosynthesis. *Annu. Rev. Plant Physiol.* <https://doi.org/10.1146/annurev.pl.33.060182.001533>.
- Fatima, S., Hameed, M., Ahmad, F., Ahmad, M.S.A., Khalil, S., Munir, M., Ashraf, M., Ahmad, I., Ahmad, K.S., Kaleem, M., 2022. Structural and functional responses in widespread distribution of some dominant grasses along climatic elevation gradients. *Flora Morphol. Distrib. Funct. Ecol. Plants.* <https://doi.org/10.1016/j.flora.2022.152034>.
- Gerendás, J., 1999. Significance of nickel for plant growth and metabolism. *J. Plant Nutr. Soil Sci.* [https://doi.org/10.1002/\(SICI\)1522-2624\(199906\)162:3<241::AID-JPLN241>3.0.CO;2-Q](https://doi.org/10.1002/(SICI)1522-2624(199906)162:3<241::AID-JPLN241>3.0.CO;2-Q).
- Gonnelli, C., Renella, G., 2012. Chromium and nickel. In: Alloway, B.J. (Ed.), *Heavy Metals in Soils*. Springer, Dordrecht, pp. 313–333.
- Hartvig, P., 2002. *Leptoplax*. In: Strid, A., Tan, K. (Eds.), *Flora Hellenica*, 2. ARG Gantner Verlag, Ruggell, pp. 231–232.
- Hoagland, D., Arnon, D.I., 1950. The water-culture method for growing plants without soil the college of agriculture. *Calif. Agric. Exp. Stn. Circ.*
- James, R.A., Munns, R., Von Caemmerer, S., Trejo, C., Miller, C., Condon, T., 2006. Photosynthetic capacity is related to the cellular and subcellular partitioning of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> in salt-affected barley and durum wheat. *Plant, Cell Environ.* <https://doi.org/10.1111/j.1365-3040.2006.01592.x>.
- Kachenko, A.G., Singh, B., Bhatia, N.P., Siegel, R., 2008. Quantitative elemental localisation in leaves and stems of nickel hyperaccumulating shrub *Hybanthus floribundus* subsp. *floribundus* using micro-PIXE spectroscopy. *Nucl. Instrum. Methods Phys. Res. Sect. B Beam Interact. Mater. At.* <https://doi.org/10.1016/j.nimb.2007.11.053>.
- Kazakou, E., Dimitrakopoulos, P.G., Baker, A.J.M., Reeves, R.D., Troumbis, A.Y., 2008. Hypotheses, mechanisms and trade-offs of tolerance and adaptation to serpentine soils: From species to ecosystem level. *Biol. Rev.* <https://doi.org/10.1111/j.1469-185X.2008.00051.x>.
- Kidd, P.S., Bani, A., Benizri, E., Gonnelli, C., Hazotte, C., Kissler, J., Konstantinou, M., Kuppens, T., Kyrkas, D., Laubie, B., Malina, R., Morel, J.L., Olcay, H., Pardo, T., Pons, M.N., Prieto-Fernández, Á., Puschenreiter, M., Quintela-Sabaris, C., Ridard, C., Rodríguez-Garrido, B., Rosenkranz, T., Rozpadek, P., Saad, R., Selvi, F., Simonnot, M.O., Tognacchini, A., Turnau, K., Wazny, R., Witters, N., Echevarria, G., 2018. Developing sustainable agronomy systems in agricultural ultramafic soils for nickel recovery. *Front. Environ. Sci.* <https://doi.org/10.3389/fenvs.2018.00044>.
- Krämer, U., Smith, R.D., Wenzel, W.W., Raskin, I., Salt, D.E., 1997. The role of metal transport and tolerance in nickel hyperaccumulation by *Thlaspi goesingense* Halacsy. *Plant Physiol.* <https://doi.org/10.1104/pp.115.4.1641>.
- Lazzaro, L., Colzi, I., Ciampi, D., Gonnelli, C., Lastrucci, L., Bazihizina, N., Viciani, D., Coppi, A., 2021. Intraspecific trait variability and genetic diversity in the adaptive strategies of serpentine and non-serpentine populations of *Silene paradoxa* L. *Plant Soil.* <https://doi.org/10.1007/s11104-020-04780-1>.
- Lucisine, P., Echevarria, G., Sterckeman, T., Vallance, J., Rey, P., Benizri, E., 2014. Effect of hyperaccumulating plant cover composition and rhizosphere-associated bacteria on the efficiency of nickel extraction from soil. *Appl. Soil Ecol.* <https://doi.org/10.1016/j.apsoil.2014.04.011>.
- Maestri, E., Marmiroli, M., Visioli, G., Marmiroli, N., 2010. Metal tolerance and hyperaccumulation: costs and trade-offs between traits and environment. *Environ. Exp. Bot.* <https://doi.org/10.1016/j.envexpbot.2009.10.011>.
- Magnani, F., Borghetti, M., 1995. Interpretation of seasonal changes of xylem embolism and plant hydraulic resistance in *Fagus sylvatica*. *Plant. Cell Environ.* <https://doi.org/10.1111/j.1365-3040.1995.tb00570.x>.
- Marschner, H., 1995. Mineral nutrition of higher plants. *Miner. Nutr. High. Plants.* <https://doi.org/10.1016/b978-0-12-473542-2.x5000-7>.
- Maxwell, K., Johnson, G.N., 2000. Chlorophyll fluorescence - a practical guide. *J. Exp. Bot.* <https://doi.org/10.1093/jxb/51.345.659>.

- Mesjasz-Przybyłowicz, J., 2001. Nuclear microprobe studies of elemental distribution in apical leaves of the Ni hyperaccumulator *Berkheya coddii*. *Serpent.-. Ecol. Third Int. Conf. Serpent.-. Ecol.*
- Mesjasz-Przybyłowicz, J., Przybyłowicz, W.J., 2011. PIXE and metal hyperaccumulation: From soil to plants and insects. *X-Ray Spectrom.* <https://doi.org/10.1002/xrs.1304>.
- Minguzzi, C., Vergnano, O., 1948. Il contenuto di nichel nelle ceneri di *Alyssum bertolonii* Desv. *Atti Della Soc. Toscan. di Sci. Nat. Ser. A* 55, 49–77.
- Montargès-Pelletier, E., Chardot, V., Echevarria, G., Michot, L.J., Bauer, A., Morel, J.L., 2008. Identification of nickel chelators in three hyperaccumulating plants: an X-ray spectroscopic study. *Phytochemistry*. <https://doi.org/10.1016/j.phytochem.2008.02.009>.
- Ouyang, W., Struik, P.C., Yin, X., Yang, J., 2017. Stomatal conductance, mesophyll conductance, and transpiration efficiency in relation to leaf anatomy in rice and wheat genotypes under drought. *J. Exp. Bot.* <https://doi.org/10.1093/jxb/erx314>.
- Pardo, T., Rodríguez-Garrido, B., Saad, R.F., Soto-Vázquez, J.L., Loureiro-Viñas, M., Prieto-Fernández, Á., Echevarria, G., Benizri, E., Kidd, P.S., 2018. Assessing the agromining potential of Mediterranean nickel-hyperaccumulating plant species at field-scale in ultramafic soils under humid-temperate climate. *Sci. Total Environ.* <https://doi.org/10.1016/j.scitotenv.2018.02.229>.
- Pignattelli, S., Colzi, I., Buccianti, A., Cecchi, L., Arnetoli, M., Monnanni, R., Gabbriellini, R., Gonnelli, C., 2012. Exploring element accumulation patterns of a metal excluder plant naturally colonizing a highly contaminated soil. *J. Hazard. Mater.* 227–228. <https://doi.org/10.1016/j.jhazmat.2012.05.075>.
- Pollard, A.J., Reeves, R.D., Baker, A.J.M., 2014. Facultative hyperaccumulation of heavy metals and metalloids. *Plant Sci.* <https://doi.org/10.1016/j.plantsci.2013.11.011>.
- Posch, S., Bennett, L.T., 2009. Photosynthesis, photochemistry and antioxidative defence in response to two drought severities and with re-watering in *Allocauarina luehmannii*. *Plant Biol.* <https://doi.org/10.1111/j.1438-8677.2009.00245.x>.
- Psaras, G.K., Constantinidis, T., Cotsopoulos, B., Manetas, Y., 2000. Relative abundance of nickel in the leaf epidermis of eight hyperaccumulators: Evidence that the metal is excluded from both guard cells and trichomes. *Ann. Bot.* <https://doi.org/10.1006/anbo.2000.1161>.
- Redjala, T., Sterckeman, T., Skiker, S., Echevarria, G., 2010. Contribution of apoplast and symplast to short term nickel uptake by maize and *Leptoplax emarginata* roots. *Environ. Exp. Bot.* <https://doi.org/10.1016/j.envexpbot.2009.10.010>.
- Reeves, R.D., Brooks, R.R., Press, J.R., 1980. Nickel accumulation by species of *Peltaria* jacq. (Cruciferae). *Taxon.* <https://doi.org/10.2307/1220334>.
- Reeves, R.D., Baker, A.J.M., Jaffré, T., Erskine, P.D., Echevarria, G., van der Ent, A., 2018. A global database for plants that hyperaccumulate metal and metalloid trace elements. *N. Phytol.* <https://doi.org/10.1111/nph.14907>.
- Resetnik, I., Schneeweiss, G.M., Liber, Z., 2014. Two new combinations in the genus *Bormuelleria* (Brassicaceae). *Phytotaxa.* <https://doi.org/10.11646/phytotaxa.159.4.8>.
- Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-response analysis using R. *PLoS One* 10. <https://doi.org/10.1371/journal.pone.0146021>.
- Scartazza, A., Di Baccio, D., Mariotti, L., Bettarini, I., Selvi, F., Pazzagli, L., Colzi, I., Gonnelli, C., 2022. Photosynthesizing while hyperaccumulating nickel: Insights from the genus *Odontarrhena* (Brassicaceae). *Plant Physiol. Biochem.* <https://doi.org/10.1016/j.plaphy.2022.02.009>.
- Selvi, F., Carrari, E., Colzi, I., Coppi, A., Gonnelli, C., 2017. Responses of serpentine plants to pine invasion: Vegetation diversity and nickel accumulation in species with contrasting adaptive strategies. *Sci. Total Environ.* 595 <https://doi.org/10.1016/j.scitotenv.2017.03.249>.
- Sharkey, T.D., 2016. What gas exchange data can tell us about photosynthesis. *Plant Cell Environ.* <https://doi.org/10.1111/pce.12641>.
- van der Ent, A., Baker, A.J.M., Reeves, R.D., Pollard, A.J., Schat, H., 2013. Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. *Plant Soil.* <https://doi.org/10.1007/s11104-012-1287-3>.
- Van Der Ent, A., Baker, A.J.M., Reeves, R.D., Chaney, R.L., Anderson, C.W.N., Meech, J. A., Erskine, P.D., Simonnot, M.O., Vaughan, J., Morel, J.L., Echevarria, G., Fogliani, B., Rongliang, Q., Mulligan, D.R., 2015. Agromining: farming for metals in the future? *Environ. Sci. Technol.* <https://doi.org/10.1021/es506031u>.
- van der Pas, L., Ingle, R.A., 2019. Towards an understanding of the molecular basis of nickel hyperaccumulation in plants. *Plants.* <https://doi.org/10.3390/plants8010011>.
- Wang, H., Prentice, I.C., Davis, T.W., Keenan, T.F., Wright, I.J., Peng, C., 2017. Photosynthetic responses to altitude: an explanation based on optimality principles. *N. Phytol.* <https://doi.org/10.1111/nph.14332>.
- Wellburn, A.R., 1994. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *J. Plant Physiol.* [https://doi.org/10.1016/S0176-1617\(11\)81192-2](https://doi.org/10.1016/S0176-1617(11)81192-2).
- Zhang, X., Houzelot, V., Bani, A., Morel, J.L., Echevarria, G., Simonnot, M.O., 2014. Selection and combustion of Ni-hyperaccumulators for the phytomining process. *Int. J. Phytoremediat.* <https://doi.org/10.1080/15226514.2013.810585>.