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SPECIAL FEATURE

Ultramafic Ecology: Proceedings of the 10th International Conference on Serpentine Ecology

Phenotyping of the nickel metal crop *Bornmuellera emarginata* for establishing breeding selection criteria

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Abstract

Nickel is a crucial metal widely used in various industrial applications, such as lithium-ion batteries and stainless-steel production. The increasing demand for nickel and resource depletion challenges highlight the need for sustainable extraction methods. Agromining employs hyperaccumulator plants to extract nickel from soils that are either contaminated or naturally enriched in nickel. *Bornmuellera emarginata* is a nickel hyperaccumulator originating from Greece and a promising candidate for nickel agromining. This study aimed to identify suitable genotypes for a successful “domestication” breeding program, focusing on phenotypic traits. Seeds from 35 plants across six wild populations were cultivated under controlled conditions for 7 months to complete a full life cycle. In this period, growth parameters (e.g., plant height (PH), leaf size, and biomass) were determined. Geographically proximate populations displayed greater phenotypic similarity than those from distant locations. Substantial morphological variations, including PH (40–126 cm) and leaf length (6–13 cm), were observed. Nickel concentrations in plants ranged from 290 to 6250 mg kg⁻¹ dry weight, resulting in nickel yields of 10–145 mg/plant. The notable phenotypic differences among genotypes provide a valuable biological resource for initiating a breeding program to enhance overall nickel yield in *B. emarginata* agromining crops. Selection and breeding efforts should prioritize genotypes with high biomass production and nickel concentrations. This research lays the foundation for targeted breeding to optimize the potential of *B. emarginata* in nickel agromining.

KEYWORDS

breeding, domestication, hyperaccumulator, nickel, phytomining

1 | INTRODUCTION

Due to their sessile nature, plants are generally well equipped to withstand a wide range of environmental

conditions. Some species are highly adapted to grow under extreme conditions. A small group of plant species, known as metal hyperaccumulators, can survive at sites that are naturally enriched or anthropogenically contaminated

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with toxic metals, often even without showing symptoms of toxicity (Brooks et al., 1977). Various hypotheses have been proposed for this ability, among the most plausible is to confer resistance to herbivores and pathogens (Boyd & Martens, 1992). Hyperaccumulation is a model trait used to study the molecular mechanisms and physiology of plant adaptations to extreme environmental conditions (Kraemer, 2010). It has been exploited for the remediation of contaminated soil, in what is known as phytoextraction (Chaney, 1983). A variant of this approach, is to use metal hyperaccumulator species for phytomining and agromining to obtain valuable metals (Chaney, 1983; Nkrumah et al., 2016; van der Ent et al., 2015). Phytomining comprises the extraction of target metals from a metal-rich soil using hyperaccumulating plants (then designated as “metal crops”) (Chaney et al., 1997). Agromining involves the complete value-chain of commercial phytomining operations, incorporating optimized germplasm and agronomy to make economically the most efficient use of phytomining (van der Ent et al., 2015). Nickel (Ni) agromining could become a new opportunity for modern industries in the context of the global climate crisis, to supply in the growing demand for Ni for lithium-ion (Li-ion) battery production with an emphasis on the decarbonization of the metal supply. The demand for Ni-containing batteries is continually on the rise, with current Li-ion batteries already containing 39% Ni, a proportion projected by the Ni producing industry to increase to 58% by 2030 (<https://nickelinstitute.org>, Nickel in Batteries, n.d.).

Nickel hyperaccumulator plants have been defined as species capable of accumulating more than 1000 mg kg⁻¹ of Ni in dry biomass (Reeves et al., 2018; van der Ent et al., 2015). Currently, more than 500 Ni hyperaccumulator species are known globally (Reeves et al., 2018). Although Ni hyperaccumulators have gained significant attention for their potential use in agromining applications, such as those described by Bani et al. (2015), Nkrumah (2018), and Nkrumah et al. (2016, 2022), there is a lack of research on their targeted breeding for application in Ni agromining. Most studies have focused on identifying new hyperaccumulator species, investigating their ecophysiology, and demonstrating the genetic differences in metal uptake and biomass production (Bhargava et al., 2012). Breeding for domestication of these species will be essential to develop economically viable agromining applications. It is a time-consuming process though, as demonstrated by the lengthy process involved in eliminating seed-shattering, one of the undesired wild characteristics that needs to be removed for successful cultivation of seed crops (Gepts, 2014; Purugganan & Fuller, 2009).

The genetic improvement of plant traits through breeding is most efficient if there is a deep understanding of the genetic mechanisms underlying such traits. To

develop a Ni agromining crop, thorough knowledge of the Ni hyperaccumulation and Ni tolerance characteristics is crucial. Genetic diversity can be harnessed to improve plant genotypes and phenotypes for successful phytomining (Li et al., 2003). Previous studies demonstrated there is ample genetic variation in metal uptake, sequestration and tolerance between and within hyperaccumulator species (Halimaa et al., 2014; Hanikenne & Nouet, 2011; Kraemer, 2010; Meier et al., 2018; Merlot et al., 2014), which suggests there is enough opportunity to breed for superior hyperaccumulation crop traits. However, several wild characteristics, not related to metal hyperaccumulation or tolerance, will need to be overcome to develop a domesticated agromining crop. For such, plants will need to germinate and grow uniformly, in high density, with the right plant architecture to maximize metal-containing biomass yield upon mechanical harvesting. Plant propagation often needs to be optimized to ensure sufficient high-quality plant material for the next season.

To date, only two studies have been conducted on breeding hyperaccumulator species with the aim of improving cadmium (Cd) or Ni phytoextraction using *Noccaea caerulea* or two *Odontarrhena* (*Alyssum*) species (Li et al., 2003; Sterckeman et al., 2019). Sterckeman et al. (2019) selected for improved shoot biomass production and Cd accumulation capacity of *N. caerulea* under Cd-contaminated soil conditions. The traits leading to high biomass production were not yet fixed in the F2 to F3 generation because of the high complexity of the trait. Li et al. (2003) significantly increased shoot Ni concentration and Ni yield by using genetically diverse Ni hyperaccumulator species and ecotypes of *Odontarrhena chalcidica* (formerly *Alyssum murale*) and *O. corsica* (formerly *A. corsicum*) in combination with appropriate soil management (Kidd et al., 2018). Plant lines were also selected for leaf retention during early flowering to avoid the loss of high-Ni foliar biomass before harvest. Leaves contribute significantly to the phytoextraction yield, as they contain Ni concentrations that are 3- to 4-fold higher than in stems.

Bornmuellera emarginata (Boiss.) Rešetnik (syn. *Lepotolax emarginata* (Boiss.) O. E. Schulz) (*Brassicaceae*; biannual or perennial), is a Ni hyperaccumulator endemic to ultramafic soils in Greece. It is distributed discontinuously from Euboea Island to the Pindus Mountains, as well as on Mt. Smolikas (Chardot et al., 2005). *Bornmuellera emarginata* is a moderately large plant, with some specimens reaching up to two meters in height (Chardot et al., 2005; Tutin et al., 1964). It is considered a good candidate for Ni agromining because of its high Ni foliar concentrations, which can reach up to 34,890 mg kg⁻¹ (Jakovljević et al., 2022).

The aim of this study was to assess the phenotypic variation of *B. emarginata* found between different genotypes, which can be exploited to improve this “metal crop” for the full implementation of Ni agromining. To this end, six populations from different regions in Greece, covering the environmental range of the species, were collected, and phenotypically characterized in pot experiments based on characteristics such as plant height (PH), leaf length (LL) and width, and Ni accumulation.

2 | MATERIALS AND METHODS

2.1 | Samples collection and preparation

Six populations of *B. emarginata* were visited at the end of July 2021 from different regions in Greece: (Agios Stefanos [AS], 39°02′04.5″ N 22°14′08.0″ E), (Kedros [CD], 39°12′28.1″ N 22°02′46.6″ E), (Mantoudi [MT], 38°46′14.7″ N 23°28′27.7″ E), (Metsovo [MV], 39°50′15.8″ N 21°05′13.6″ E), Milia (ML, 39°51′13.5″ N 21°14′02.8″ E), (Pefki [PF], 39°47′36.02″ N 21°22′52.59″ E). Seeds were collected from individual plants from the different sites, each containing one population. The sampling sites covered the geographical range of the species, that is, from Euboea Island to Pindus National Park (Figure 1). Seeds were collected from different plants of each population and combined. To complete the ripening process, the seeds were first kept at 30°C for 1 month in a greenhouse in Nancy (France). At the end of the ripening process, seeds were tested for germination in Petri dishes. As seeds were collected from multiple plants in each population, and thereafter bulked, the plants that were grown from the germinated seeds should all be considered different genotypes. Given the relative isolation of the populations, we expect plants from the same population to be more related than plants from different populations. For each population at least three plants could be grown from the germinated seeds. All plants were propagated in the same conditions, using regular, potting mix as substrate, not on ultramafic soil, to ensure comparable growth conditions for all genotypes. Based on the outcome of the germination tests, varying quantities of seeds from each population were sown in potting soil. These seeds were allowed to germinate for 1 week before single healthy seedlings were transplanted into 3-L pots. The seedlings correspond to two times 5, 4, 12, 6, and three genotypes for the AS, CD, ML, MT, MV, and PF populations, respectively. They were grown for 8 weeks under controlled conditions with settings at a light intensity of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a temperature of 22°C day/15°C night, a photoperiod of 18 h, and a relative air humidity of 70%. The plants were watered twice a week with Terra Vega nutrient (<https://www.canna.fr/canna-terra>) solution

diluted 1:2500 with deionized water, to stimulate growth. After 2 months, the plants were subjected to cold vernalization in a growth chamber with the same parameters, with a reduced temperature of 5°C and a photoperiod of 10 h, for 6 weeks. At the end of the vernalization, the conditions of the growth chamber were restored to the conditions of the growing period, to initiate flowering and allow plants to complete their life cycle for a period of 7 months. One week after vernalization, 250 mg kg⁻¹ NiSO₄ was added to each pot, from a stock solution of 100 mM.

2.2 | Soil characteristics of the population localities

Soil samples were collected from the top 1–10 cm at the site where each plant was sampled (Figure 1). After collection, the soils were air-dried and passed through a 2-mm stainless steel mesh sieve. The pH of each soil sample was measured using a 1:5 soil to water ratio, with 1 h of agitation. To extract the exchangeable trace elements, 10 mL of diethylenetriaminepentaacetic acid (DTPA) solution was added to 5 g of each soil sample (Lindsay & Norvell, 1978). The samples were shaken for 2 h and then filtered with a non-sterile PTFE Syringe Filter (Fisherbrand™) before being analyzed by ICP-OES (ThermoFisher, iCAP 6000 DUO model Emission Spectrometer).

2.3 | Phenotypic characterization

The morpho-agronomic characteristics of *B. emarginata* PH, LL, leaf width (LW), dry biomass (DB), and branch number (BN) were recorded on plants grown from collected seeds. The PH was measured from the collar to the tip of the most extended leaf. LL and LW were recorded as described by Verwijst and Wen (1996). Three leaves (the 15th, 20th, and 25th from the basis) were selected for the measurements. LL was measured from petiole to the lamina tip and LW was measured as the maximum LW (Figure 2). Then, the final value of the leaf characteristics was determined by calculating the average of the three measurements. The BN of the plants were recorded by counting all the branches present on each plant (Figure 2). At the end of the experiment, when plants had flowered and set seed, entire plants were harvested, meticulously rinsed with deionized water, and subsequently divided into the main stem, which was designated “stem” and the rest of the above-ground matter, consisting mainly of leaves, but also including small side twigs, flowers, and fruits, which was termed “leaves” for simplicity. The DB was

Sampling Sites in Greece

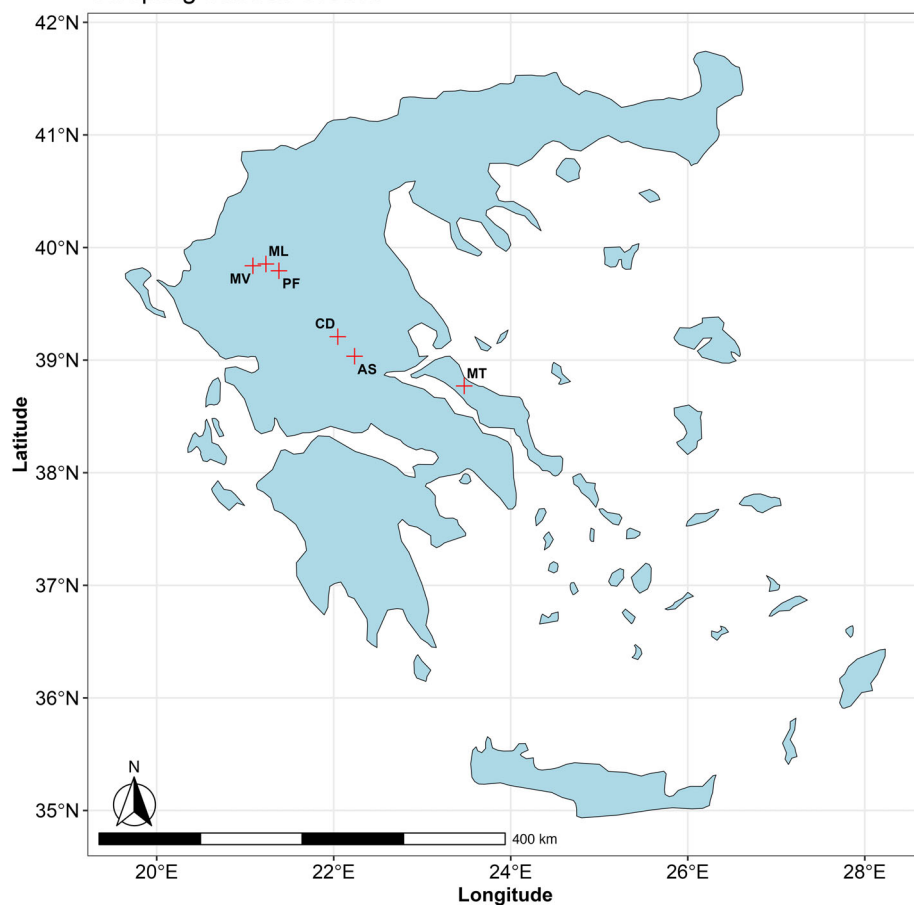


FIGURE 1 Geographical locations of collection sites: The geographical locations for the plant and soil collection sites of the six populations of *Bornmuellera emarginata* in Greece are indicated. A cross symbol is used to mark each sampling site for all the genotypes. For GPS coordinates, see Section 2. AS, Agios Stefanos; CD, Kedros; ML, Milia; MT, Mantoudi; MV, Metsovo; PF, Pefki. Source: The R software version 4.3.1 (June 16, 2023).

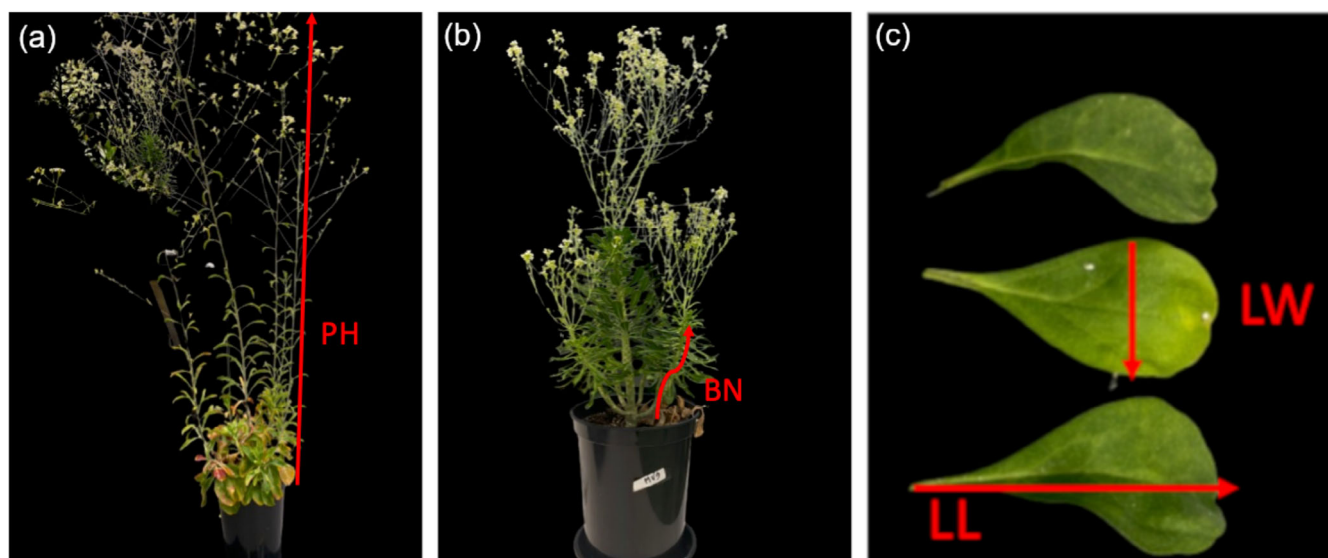


FIGURE 2 *Bornmuellera emarginata* plants during vegetative growth in a controlled environment. Plants are growing on a peat-based common garden potting soil with $250 \text{ mg kg}^{-1} \text{ NiSO}_4$. (a) Plant corresponding to a genotype from the Mantoudi population; (b) genotype from Metsovo population; (c) cut leaves for measurement. BN, branch number; LL, leaf length; LW, leaf width; PH, plant height.

determined on all the above-ground parts (stem and leaves). We therefore also calculated the Ni yield of each genotype, which is the total amount of harvestable Ni

per plant (in mg), based on the Ni concentrations from the averages of leaves and stem measurement and the dry above-ground plant biomass.

2.4 | Elemental analysis of plants

At the end of the experiment, the Ni concentration in the plants was measured by grinding the entire dry stem and leaves samples separately, then 0.5 g was digested with 5 mL HNO_3 and 2.5 mL H_2O_2 . These traits of Ni accumulation are termed as follows: Stem Ni (SNi) concentration; Leaf Ni (LNi) concentration; Nickel Yield (NY). After cold digestion for 16 h, the samples were placed in a DigiPREP system for hot (at 125°C) digestion for 180 min to be filtered before ICP-OES analysis (ThermoFisher, iCAP 6000 DUO).

2.5 | Statistical analysis

The phenotypic data were subjected to statistical analysis using the R software version 4.3.1 (June 16, 2023). Inter-population and intra-population comparisons were performed. The mean of each population was calculated and visualized using a boxplot. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test at a 95% confidence level ($p < 0.05$) was performed when the normality was confirmed by the Shapiro–Wilk test. Kruskal–Wallis was performed when the normality was not attended. Pearson's linear correlation and principal component analysis (PCA) were performed to determine the

origin of variation using the data on all eight measured traits, including morphological traits and Ni accumulation traits. The Euclidean distance tool was used to establish the complete linkage distance between the different genotypes.

3 | RESULTS

3.1 | Sampling of *B. emarginata* genotypes

Six populations of *B. emarginata*, from six different locations on ultramafic soils in Greece (Figures 1 and 3), were sampled. Plants varied considerably in terms of morphology and stage of development. Not all plants carried ripe seeds (Figure 3). DTPA-extraction of the soil was used to provide information on the levels of metals that were available for plant uptake. The soil characteristics at the different sampling sites showed significant variability in the soil DTPA-extractable Ni from site to site, ranging from 31.6 to 218 mg kg^{-1} (Table 1). The highest value was found at the PF site, while the lowest value was found at the AS site. Soil pH was not significantly different and ranged between 6.5 and 7.1. Concentrations of other metals also varied, most for Zn (0.51–13.89 mg kg^{-1}), least for Mg (485–1200 mg kg^{-1}).



FIGURE 3 *Bornmuellera emarginata* at different stages in the field. (a) Mature plant in its natural environment; (b) shoot of mature plant; (c) young siliques; (d) mature siliques with ripe seeds.

TABLE 1 List of populations, their soil characteristics, and seed germination rates for each of the sampled populations.

Region	Population (SC)	Soil pH	DTPA extractable metal concentration (mg kg ⁻¹)						GR %
			Ni	Cu	Co	Mg	Zn	Mn	
Phthiotis	Agios Stefanos (AS)	6.59	31.6	0.52	0.12	835	0.51	4.35	56
Karditsa	Kedros (CD)	6.60	45.1	0.27	0.24	531	0.96	7.49	44
Ioannina	Milia (ML)	6.86	71.4	0.95	0.48	1204	1.25	21.24	4
Euboea	Mantoudi (MT)	7.04	100	0.38	0.56	1009	1.89	14.89	84
Ioannina	Metsovo (MV)	6.64	117	0.38	0.53	636	1.47	15.21	4
Trikala	Pefki (PF)	6.56	218	0.42	0.82	485	13.89	26.77	12

Note: Values represent the mean of samples.

Abbreviations: DTPA, diethylenetriaminepentaacetic acid; GR%, germination percentage; SC, samples code.

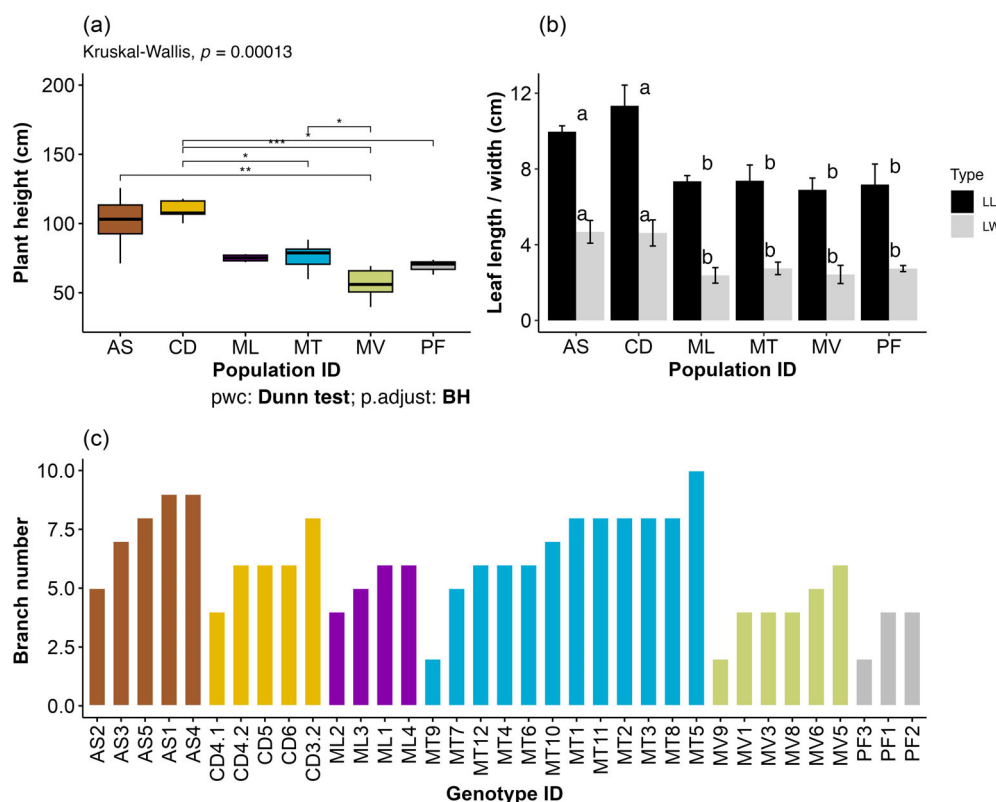


FIGURE 4 Plant characteristics after 12 weeks of growth. (a) Boxplot of plant height (PH), (b) average leaf length (LL) and leaf width (LW) of the six populations. (c) The branch number of all genotypes. Differences between populations are indicated with an asterisk for (a) and with lowercase letters for (b) ($p < 0.05$). AS, Agios Stefanos; CD, Kedros; ML, Milia; MT, Mantoudi; MV, Metsovo; PF, Pefki.

3.2 | Growth parameters of the *B. emarginata* genotypes

We conducted germination tests in Petri dishes to evaluate the germination potential of the seeds collected from the field. The germination rates varied substantially among the sampled populations (Table 1). The population from MT exhibited the highest germination rate, with 84% of the seeds successfully germinating. In contrast, the populations from Melia (ML) and MV displayed substantially lower germination rates, only around 4%. PH differed significantly between the six populations (Figures 2 and 4a). One plant from AS reached 126 cm,

while one plant from the MV population remained only 40 cm. The LL ranged from 6.0 cm in PF to 13.0 cm in CD. LW ranged from 1.6 cm in MV to 5.4 cm in AS (Figures 2–4b). The number of branches (BN) varied between the genotypes. Plants from MT presented the highest BN with an average of 10 while the lowest count averaged of 2 was recorded in the population from PF (Figure 4c). The total above-ground DB (Figure 5) showed a wide variation within and between populations, but no significant differences were observed between populations. The highest biomass was recorded for Agios Stefanos 1 (AS1), at 58.3 g DW, whereas the lowest was for Metsovo (MV1) with 3.8 g of biomass

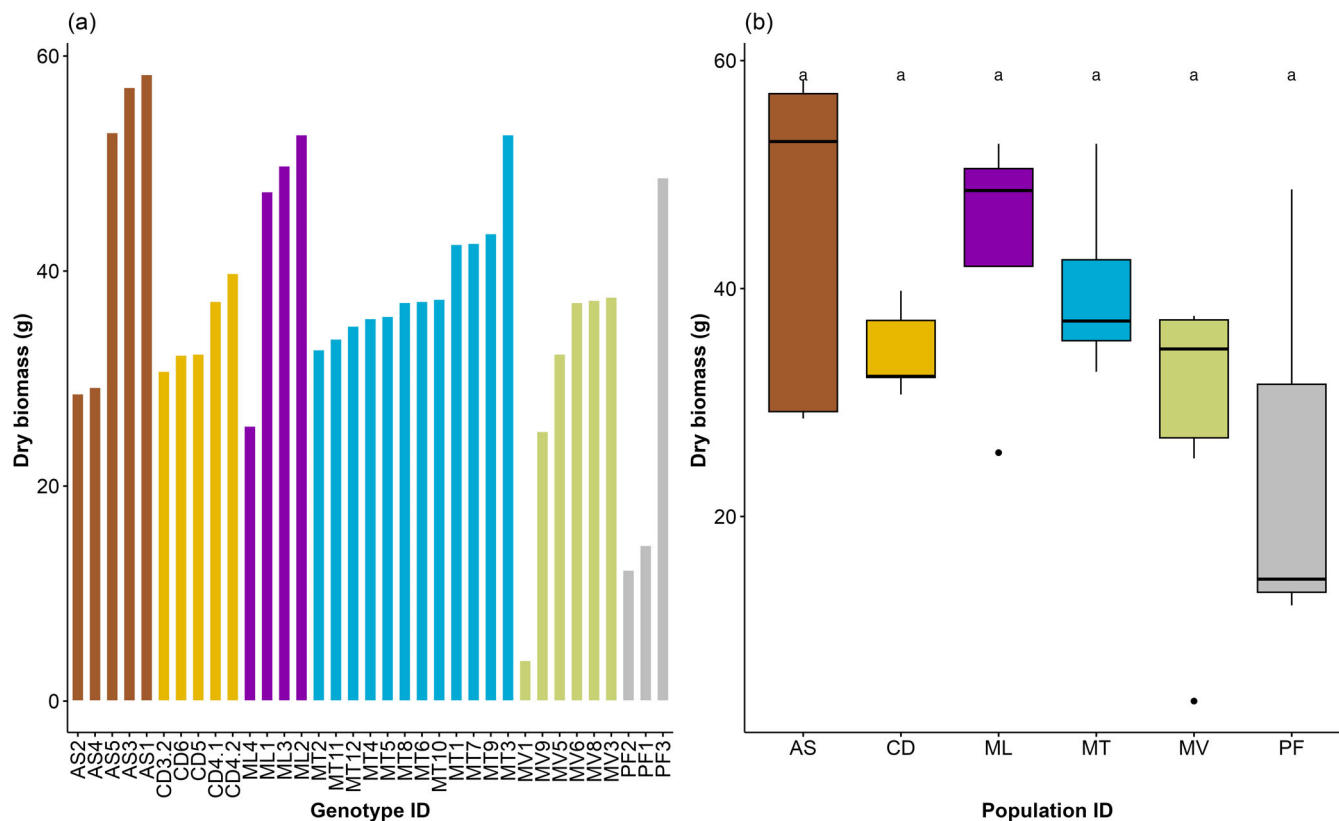


FIGURE 5 Variation for plant total dry biomass (above-ground) per plant within and between *Bornmuellera emarginata* populations. (a) Dry biomass for each genotype; (b) box plots of dry biomass at the population level. Biomass was determined at the end of the experiment. AS, Agios Stefanos; CD, Kedros; ML, Milia; MT, Mantoudi; MV, Metsovo; PF, Pefki.

(Figure 5), which means more than 15-fold difference between the plants with the lowest and the highest DB.

3.3 | Plant nickel concentrations and nickel yields of the *B. emarginata* genotypes

The Ni concentrations for most genotypes exceeded the Ni hyperaccumulator threshold of $1000 \text{ mg Ni kg}^{-1}$ dry weight in their leaves, thereby confirming their hyperaccumulator status (Figure 6a). Only some genotypes of the AS and MT populations did not reach the hyperaccumulator threshold. The highest concentration was found in the MV1 genotype, reaching 6250 mg kg^{-1} , the lowest was found in the MT4 genotype (240 mg kg^{-1}) (Figure 6a). The differences in Ni accumulation in leaves and stems between the different genotypes were statistically significant at the population level, but only for two populations that differed from the rest (MT and MV), with MT showing the lower range of values and MV showing the higher range of values (Figure 7). Nickel concentration followed the same trend in both parts, that is, leaves and stem of the plants. In general, the Ni concentration in leaves is negatively correlated with

the total DB of the plants (Pearson's correlation coefficient $r = -0.47$; Figure 8a), meaning that larger plants have relatively lower Ni concentrations. This showed large variation among the MT and PF genotypes, and only little variation among the AS and CD genotypes. The genotypes PF3, MT7, MT12, and ML1 exhibited the highest Ni yields, measuring 145, 143, 125, and 105 mg Ni per plant in the harvestable biomass (Figure 6b). These are the genotypes that produce a relatively large biomass at a relatively elevated concentration of accumulated Ni. There is no significant correlation between Ni concentration and Ni yield (Figure 8b), as there are also small plants with a relatively low Ni concentrations, and large plants with a relatively high Ni concentration. Not all plants produced seeds, but plants that did could be genotypes of interest to continue with for further development of an Ni agromining crop. Alternatively, other genotypes from the same population could be used.

3.4 | Principle component analysis of *B. emarginata* phenotypic traits

The six populations clustered into four distinct groups (Figure 9). One cluster comprised all genotypes of the

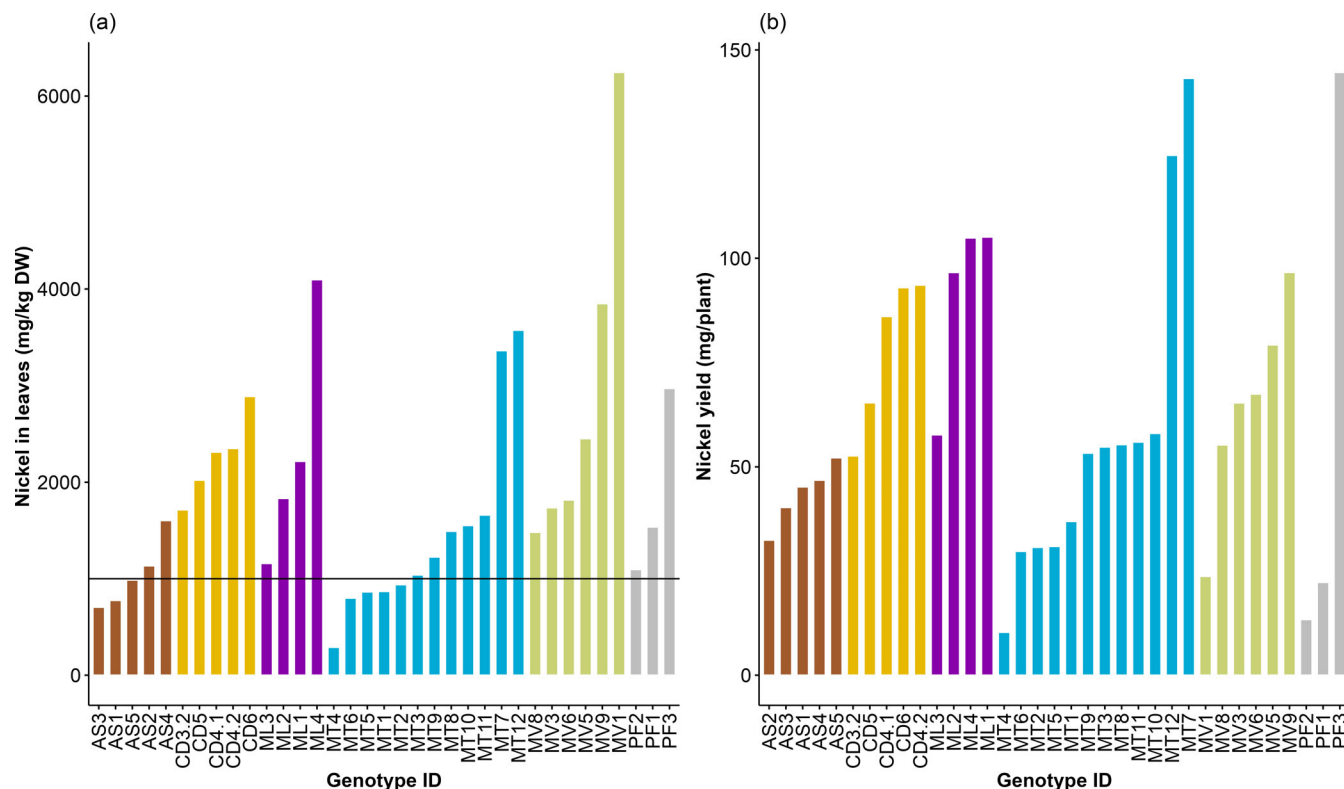


FIGURE 6 Ni concentrations and Ni yield of the 35 *Bornmuellera emarginata* genotypes tested. (a) Leaves Ni concentrations (in mg kg^{-1} dry weight [DW]), the black line shows the Ni hyperaccumulation threshold (1000 mg kg^{-1} DW); and (b) Ni yield (in mg/plant), which is the total Ni accumulated in the harvestable (aboveground) plant parts. The genotypes are indicated with population abbreviation and number. AS, Agios Stefanos; CD, Kedros; ML, Milia; MT, Mantoudi; MV, Metsovo; PF, Pefki.

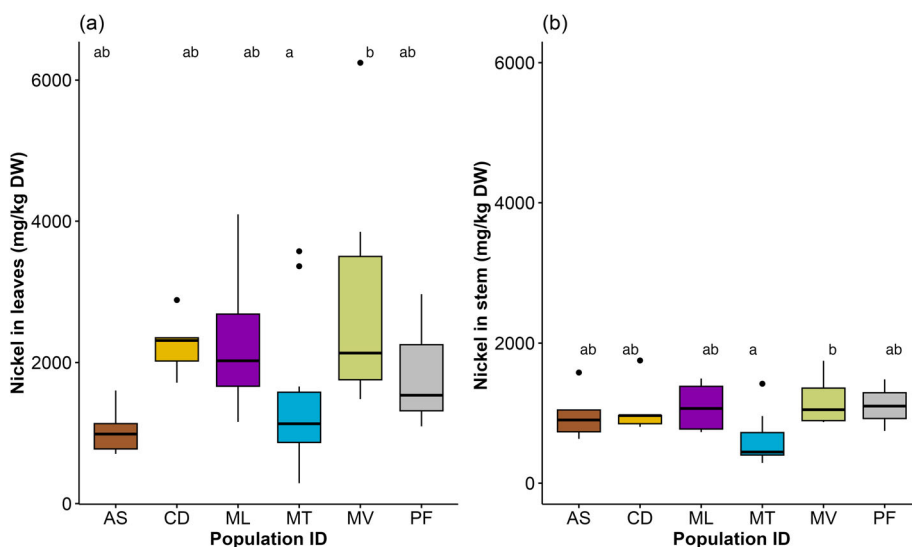


FIGURE 7 Metal concentrations in different *Bornmuellera emarginata* populations. (a) Box plots of the nickel concentration (in mg kg^{-1}) in leaves of the genotypes grown for each population; (b) Nickel concentrations in stems of the genotypes grown for each population. Differences in populations are indicated by lowercase letters ($p < 0.05$). AS, Agios Stefanos; CD, Kedros; ML, Milia; MT, Mantoudi; MV, Metsovo; PF, Pefki.

AS and CD populations, and another almost all genotypes of the MT population. The other genotypes of the ML, MV, and PF populations were grouped into two other clusters, which were less distinct than the former ones. A PCA was carried out for the eight phenotypic characteristics including morphological (PH; LL; LW; DB; BN) and Ni

accumulation traits (SNi; LNi; NY) to reveal potential relationships between traits and genotypes. The first two principal components (PCs) explained 61.3% of the total variance, with 37.1% and 24.2% of the variance on the first and second axes, respectively (Figure S1). The eigenvalues which indicate the complete extent of variability that can be

FIGURE 8 The correlation of *Bornmuellera emarginata* genotype dry aboveground biomass with Ni accumulation traits. (a) The relationship between the Ni concentration in aboveground biomass (nickel leaves, in mg kg^{-1}) and dry biomass (g); (b) the relationship between Ni yield (in mg/plant) and dry biomass (g). R is the Pearson's correlation coefficient and p is the significance level of the t -test.

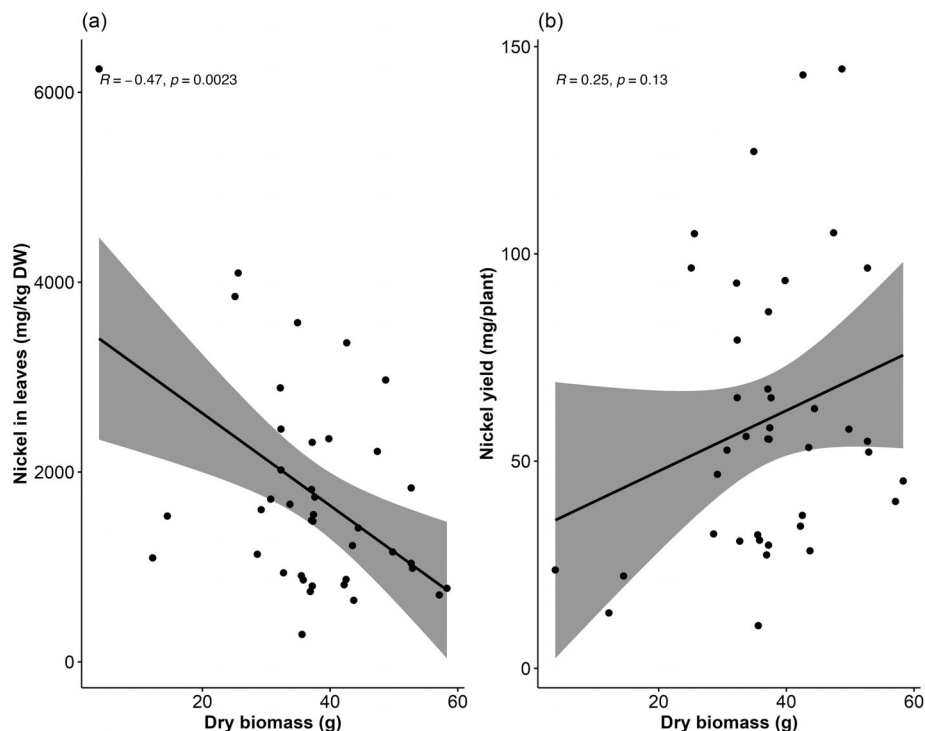
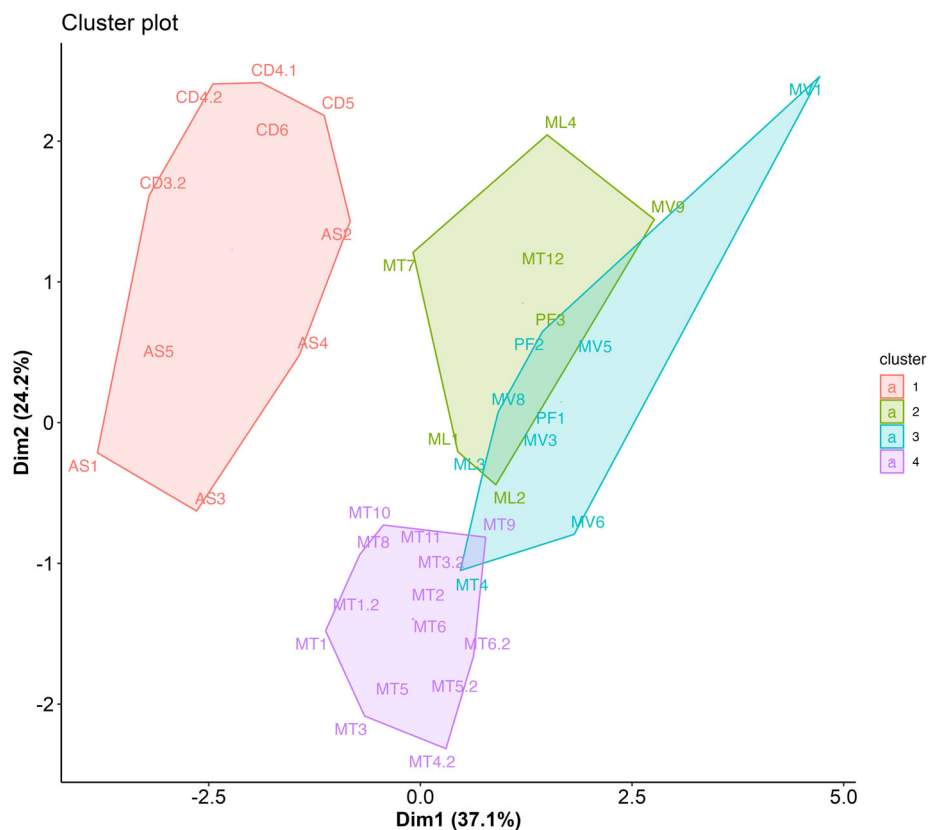


FIGURE 9 Population clustering by using the mean value of the eight measured traits (BN, branch number; DB, dry biomass; LL, leaf length; LNi, leaf nickel; LW, leaf width; NY, nickel yield; PH, plant height; SNi, stem nickel) recorded. The genotypes are indicated with the 2-letter population abbreviation and a number. Four clusters are distinguished, as indicated with different colors.



clarified by a specific PC decreased from PC1 to PC3, with values of 2.96, 1.94, and 1.51, respectively (Table 2). PC1 correlated highly positively with the total DB and its

component parameters, such as LL, LW, PH, and BN. The second axis had a highly positive correlation with stem and leaf Ni concentrations, and Ni yield.

TABLE 2 Eigen values and Eigen vectors based on eight phenotypic traits.

	PC1	PC2	PC3
Eigen values	2.96	1.94	1.51
% Total variance	37.1	24.25	18.93
% Cumulative	37.1	61.36	80.29
Traits	Eigen vectors		
Leaf length (LL)	0.78	0.51	−0.19
Leaf width (LW)	0.81	0.45	−0.08
Plant height (PH)	0.87	0.28	0.07
Branch number (BN)	0.57	−0.40	−0.26
Dry biomass (DB)	0.52	−0.41	0.61
Leaf nickel concentration (LNi)	−0.51	0.71	0.23
Stem nickel concentrations (SNi)	−0.30	0.58	−0.52
Nickel yield (NY)	−0.08	0.47	0.83

4 | DISCUSSION

Efficient Ni agromining relies on the remarkable ability of hyperaccumulator plants to accumulate high concentrations of Ni while producing substantial harvestable biomass. However, hyperaccumulators are seldomly described in literature as possessing both these two sets of traits. Considering this, our study aimed to investigate the potential of *B. emarginata*, a wild subshrub Brassicaceae species, uniquely adapted to flourish in Ni-rich ultramafic soil in the Balkans, as a candidate for efficient Ni agromining. The assessment revealed phenotypic variations within and between six natural populations of *B. emarginata*, originating from different sites in Greece, covering a large part of the natural span of this Greek endemic species. As plants are hard to compare when assessed in situ, due to large differences in local environmental conditions, we decided to collect seeds of each population, and grow plants ex situ, under common, controlled conditions. This allowed an objective comparison of different genotypes. Seeds were collected from different plants at each site, which constitutes a natural population, consisting of a mix of genotypes, that would be expected to represent the variation available within the population. Germination test of genotypes collected from different sites showed high variation between populations. This variation in seed germination probably reflects more the differences in maturity stage of the harvested seeds, than the genetic differences between the populations. Even though all populations originate from ultramafic soil, we observed significant variation in the levels of DTPA extractable metals among the soils collected at

the sampling sites (Table 1). The genotypes demonstrating high Ni accumulation capacities or substantial yields usually originate from the sites with the highest soil Ni concentrations (MV1, MT7, MT12, and PF3) although PF does not show high available Ni concentrations in soil. This could be because these genotypes are best adapted to the high Ni exposure and may have evolved mechanisms enabling them to tolerate high concentrations of Ni, which would be essential for their survival on sites characterized by a high Ni availability. However, the same populations also provided genotypes with very low Ni accumulation or yield (MT4, PF1, PF2), illustrating the wide range of genetic differences that can be found in these populations. It could be a rich genetic resource for enhancing Ni accumulation traits for efficient Ni agromining.

The Ni accumulation concentrations in both leaves and stems differed significantly between the different populations, suggesting there to be genetic variation between populations. Jakovljević et al. (2022) previously reported that *B. emarginata* can accumulate up to 34,890 mg kg^{−1} of Ni in its natural habitat. While our study reported lower Ni concentrations, it is essential to consider the environmental context and ecological factors that influence Ni accumulation in wild populations of *B. emarginata*. These variations in Ni concentrations within different populations emphasize the significance of site-specific adaptations and local ecotypes (Cerdeira-Pérez et al., 2019; Tognacchini et al., 2020). The highest Ni concentration we found was 6250 mg kg^{−1} in the MV group. This is much lower than found by Reeves et al. (1980) in the field, and also lower than the values reported by Lucisine et al. (2014) and Hipfinger et al. (2022), who found leaf Ni concentrations of more than 8000 and 17,600 mg kg^{−1}, respectively, in the shoots of populations from Trigona, which are relatively close to the MV population, grown in pot supplemented with ultramafic soil. The plants sampled by Hipfinger et al. (2022) had been grown in outdoor conditions on ultramafic soil collected elsewhere, with a total mean of 1450 mg kg^{−1} (Kidd et al., 2018), which is probably closer to their natural ultramafic substrates, while we grew plants in pots containing regular potting soil supplemented with Ni (250 mg Ni kg^{−1}), which is lower than the total Ni concentration range typically found in ultramafic soil (500–3000 mg kg^{−1}) (Brooks, 1987), but probably much more bioavailable. The concentrations we found still exceed those reported by Chardot et al. (2005), who recorded 4591 mg kg^{−1} in plants from a population originating from Katara Pass. This site is located ~5 km northeast of MV, and not the same as the MV population we sampled. Those plants were cultivated in pots filled with ultramafic soil, although with a rather low total Ni concentration of only 470.5 mg kg^{−1}.

In contrast to earlier evaluations of phytomining, using different Ni hyperaccumulators from the Mediterranean region assessed under controlled conditions, significant Ni accumulation was observed in *Berkheya coddii* and *Odontarrhena chalcidica* (Robinson et al., 1999; Rue et al., 2020; Tognacchini et al., 2020). Tognacchini et al. (2020) reported a Ni shoot concentration of 20.2 g kg^{-1} in *Odontarrhena chalcidica* growing on ultramafic soil with 1.45 g kg^{-1} pseudo-total Ni, while Bani et al. (2007) observed accumulation of 9.9 mg Ni/pot filled with unfertilized ultramafic soil. The phytoextraction yield with *Odontarrhena chalcidica* on pot was much lower compared to the estimated Ni yield (145 mg/plant) found during our experiment (Bani et al., 2007).

The morphological traits varied within and between populations, with significant differences in PH, LL, and LW. The large differences in PH between plants from the AS population (maximal 125.7 cm) and those from the MV population (minimal 39.7 cm) are important. Despite these significant differences in PH, and also leaf dimensions (Figure 4b), the differences in plant biomass, which also varied substantially (from 3.8 to 58.3 g/plant) were not statistically significant at population level (Figure 5), probably due to the large variation within populations. Considering that *B. emarginata* is a bi-annual species, the timing of the harvest significantly impacts total biomass, a crucial trait for nickel agromining. To optimize biomass retention and enhance Ni agromining efficiency, harvesting should be conducted during the flowering period. This timing prevents substantial biomass loss due to leaf fall after flowering, which is essential for maintaining high Ni yield. The above-ground parts, which contain the highest concentration of Ni, should be targeted for harvest. Therefore, harvesting during the flowering stage is recommended for optimal Ni recovery. Based on the Euclidian distance of the eight measured traits (Figure 9), genotype clustering showed that the AS population was closely related to the CD population. As these two populations are also geographically closely related (Figure 1) and originating from low altitudes, this likely also reflects their genetic relatedness. The MT population is geographically most distant from the other populations and also clusters largely separately. The ML, MV, and PF populations, which originate from geographically close locations, are grouped in two clusters, but these are overlapping, suggesting they may also genetically be more closely related than to the other populations. Further genetic characterization will be needed to establish the genetic relationships within and between the populations. The first two PCs (Figure S1) played a significant role in explaining a larger portion of the overall observed variation than the other PCs following the analysis of eigenvalues (Table 2). Phenotypic

diversity studies using PC techniques must be considered, as they enable the assessment of the significance of a trait in relation to the total observed variation (Hamid et al., 2009). PC1 accounted for 37.1% of the total variation, with LL, LW, and PH being the primary contributors to the observed diversity (Figure S1; Table 2). PC2 exhibited variation patterns that were primarily influenced by traits such as nickel content in stems and leaves (SNi, LNi) (Figure S1; Table 2).

Our research has unveiled a wide-ranging phenotypic diversity in the *B. emarginata* populations we sampled. This diversity is evident not only in terms of Ni accumulation but also in various biomass-related characteristics. Given the relatively few genotypes we examined ex situ, considering the size of the populations, and our sampling method by grouping seeds of several plants per population, this is promising in terms of the genetic variation for domestication traits expected to be found in these populations. The inherent variability provides a very promising genetic resource for developing different breeding schemes aimed at creating efficient cultivars for Ni agromining. Within this intriguing spectrum, we have identified genotypes capable of simultaneously achieving high Ni accumulation and robust biomass production, resulting in high Ni yields on a “per plant” base. When extrapolating the Ni yield, when applying a planting density of four plants per square meter, as recommended for the most promising genotypes (Bani et al., 2015), this would lead to an estimated Ni crop yield of approximately $5.8 \text{ kg Ni ha}^{-1}$ which is not much but understandable considering that it derives from an extrapolation from plants grown in pots with limited Ni input to field-size scale. The first full-scale agronomic trials in Greece have led to nickel yields up to over $150 \text{ kg Ni ha}^{-1}$ (Kyrkas et al., 2023).

The genetic diversity can be exploited by selectively crossing genotypes with distinct traits relevant to Ni agromining, combining favorable alleles for enhanced performance. Since *B. emarginata* is not very self-incompatible, and able to self-pollinate upon manual pollination, breeding could initially focus on selection of inbred lines with superior characteristics. However, it may also be possible to develop an F1 hybrid breeding scheme, and make use of the expected heterosis effect in the offspring, as previously also suggested by Sterckeman et al. (2017) for breeding of *N. caerulea*. We observed a negative correlation (The Pearson's correlation coefficient $r = -0.47$) between total harvestable biomass and Ni accumulation when comparing all genotypes, hinting at a potential trade-off relationship between these crucial traits. The negative correlation is not very strong though, as also plants were found with relatively high biomass and high Ni concentrations, resulting in high Ni yields. This suggests the

successful prospect to identify even more high Ni yielding offspring when crossing genotypes with high Ni accumulation and biomass potential from different populations. Previous analysis on *Odontarrhena chalcidica* showed that the relationship between biomass production and Ni concentrations can be negative when Ni availability in soil is a limiting factor for Ni uptake or positive when Ni availability is non-limiting, when testing the same genotypes of the species in the field (Bani & Echevarria, 2019). It is therefore important to be cautious to extrapolate our findings, based on plants grown in regular potting soil supplemented with highly available Ni, to plants grown in ultramafic soil in the field, but it appears promising to be able to generate genotypes with high biomass and high Ni accumulation properties, in a breeding scheme. Such an approach can harness the additive effects (Nguyen et al., 2022) governed by the genes responsible for these traits, potentially yielding an exceptional cultivar optimized for efficient Ni agromining.

5 | CONCLUSIONS

The phenotypic characterization of *B. emarginata* populations from different sites did not reveal highly significant distinctions between populations regarding Ni-related or morphological traits. However, the populations from MT and MV exhibited notable differences in Ni concentration compared to the others; also, AS and CD showed significantly taller plants than the other populations. The high Ni yield we observed in *B. emarginata*, reaching up to 145 mg Ni/plant, from an artificial Ni source, holds great promise to further domesticate this species for efficient use in Ni agromining. While our study has shed light on the potential of *B. emarginata*, and even identified some genotypes with promising phenotypes to further select on in breeding for an agromining crop, additional research will be needed to fully characterize and exploit the genetic variation available in the wild populations for all characteristics contributing to domestication, in serpentine field conditions, not in pot experiments using common, though Ni supplemented, potting soil mixes. This will involve assessment of many more lines to be made from progeny of individual, high biomass plants of the most promising populations, to be followed with test crosses and additional line selection, aimed to combine favorable alleles in a breeding program.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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