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Nickel phytoremediation potential of the Mediterranean *Alyssoides utriculata* (L.) Medik

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HIGHLIGHTS

- The Mediterranean *Alyssoides utriculata* is characterized as a Ni hyperaccumulator.
- *A. utriculata* stores leaf Ni >1000 μg g^{-1}, with TF and BF > 1.
- The plant can survive in both serpentine and non-serpentine soils.
- *A. utriculata* has the potential for being a Mediterranean Ni phytoremediator.

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ABSTRACT

This study investigated the accumulation and distribution of nickel in the leaves and roots of the Mediterranean shrub *Alyssoides utriculata* to assess its potential use in phytoremediation of Ni contaminated soils. Total (AAS and ICP-MS) Ni, Ca and Mg contents were analyzed in the plants and related to their bioavailability (in EDTA) in serpentine and non-serpentine soils. To find the relationships between the soil available Ni and the Ni content of this species, we also evaluated possible interactions with Ca and Mg. The bioaccumulation factor (BF) and the translocation factor (TF) were determined to assess the tolerance strategies developed by *A. utriculata* and to evaluate its potential for phytoextraction or phytostabilization.

The leaf Ni is higher than 1000 μg g^{-1} which categorizes the species as a Ni-hyperaccumulator and a great candidate for Ni-phytoextraction purposes. In addition to the accumulation of Ni, the leaf Mg is also correlated with soil bioavailable concentrations. The Ca uptake and translocation were significantly lower in serpentine plants (higher Ni), as such, the leaf Ca is probably greatly influenced either by the soil's Ni or the soil Ca/Mg ratio. The BFs and TFs are strongly higher than 1 and generally did not significantly differed between plants from serpentine (higher Ni) and non-serpentine soils (lower Ni).

The present study highlights for the first time that *A. utriculata* could be suitable for cleaning Ni-contaminated areas and provides a contribution to the very small volume of data available on the potential use of native Mediterranean plant species from contaminated sites in phytoremediation technologies.

1. Introduction

Among natural metalliferous Ni-rich soils, serpentine soils are the ones where nickel represents the most abundant metal and, typically, has total concentrations of 500–8000 μg g^{-1} (Reeves and Baker, 2000; Ghaderian et al., 2007; Van der Ent et al., 2013) and bioavailable concentrations of 7 to >100 μg g^{-1} (Freitas et al., 2004; Turgay et al., 2012) depending on soil and/or analytical method.

On metalliferous soils all over the world, plants named 'metallophytes' developed various physiological tolerance mechanisms to cope with high soil metal concentrations (Baker, 1981, 1987; Baker and Walker, 1990). Specifically, some plants known as 'hyperaccumulators' actively uptake metals at the root level and translocate them to the shoot, where they can reach very high concentrations on a dry weight (DW) basis (10,000 μg g^{-1} for Zn and Mn; 1000 μg g^{-1} for Ni, Co, Cu and Pb; and 100 μg g^{-1} for Cd), while in their natural habitat (Baker and Brooks, 1989; Baker et al., 2000).

A suitable *in situ* technique, cost-effective and environmentally sustainable for removing metals like nickel from soils is represented by phytoremediation, the use of higher plants to cleanup

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soils (Salt et al., 1998; Lasat, 2000; McGrath et al., 2002; Tripathi et al., 2007; Ali et al., 2013). One phytoremediation technique consists of phytoextraction, employing hyperaccumulator plants to concentrate metals at the shoot level (Lasat, 2002; Ali et al., 2013). Care should be taken in choosing the right hyperaccumulator species for the application of phytoremediation techniques, because the introduction of alien plants may alter and disrupt indigenous ecosystems (Angle et al., 2001), and because well-known hyperaccumulator species may be unsuitable for local climate conditions (Vangronsveld et al., 2009). Therefore, one alternate option is to find native hyperaccumulator plants adapted to grow on metalliferous sites, and use them for soil remediation in the same region (Pilon-Smits and Freeman, 2006) via metal extraction (Carillo Gonzalez and Gonzalez-Chavez, 2005). Native serpentine hyperaccumulators show physiological mechanisms that convey tolerance to extremely adverse chemical soil properties (Brooks, 1987; Alexander et al., 2007) and could be fruitfully used for phytoremediation purposes.

Leaf Ni concentration of plants on serpentine soils is generally slightly elevated compared to non-serpentine plants (10–100 $\mu g\ g^{-1}$ versus 0.2–5 $\mu g\ g^{-1}$; Reeves, 1992). However, Ni phytotoxicity varies with soil bioavailable Ni and with plant species (Mizuno, 1968; Khalid and Tinsley, 1980) consisting in about 10 $\mu g\ g^{-1}$ DW in sensitive species (Kozlow, 2005), 50 $\mu g\ g^{-1}$ DW in moderately-tolerant species (Bollard, 1983; Asher, 1991) and more than 1000 $\mu g\ g^{-1}$ DW in hyperaccumulator species (Kupper et al., 2001; Pollard et al., 2002).

In temperate regions, most Ni hyperaccumulators belong to the family Brassicaceae (about 90 *taxa*). Nickel hyperaccumulation has evolved independently at least six times in Brassicaceae, most likely on serpentine soils (Krämer, 2010). More than 50 *taxa* are in the genus *Alyssum* (Minguzzi and Vergnano Gambi, 1948; Brooks and Radford, 1978; Brooks et al., 1979; Vergnano Gambi et al., 1979; Brooks, 1998; Reeves et al., 2001; Warwick et al., 2008; Cecchi et al., 2010), able to accumulate up to 3% leaf Ni (DW).

In Italy, although serpentine vegetation has been thoroughly studied (Chiarucci, 2004; Selvi, 2007; D'Amico, 2009; Marsili, 2010; D'Amico and Previtali, 2012), only two herbaceous hyperaccumulator species have been described. Those are (1) *Thlaspi caerulescens* J.Presl & C.Presl (syn. *Noccaea caerulescens* (J.Presl & C.Presl) F.K.Mey.), with 1000–30,000 $\mu g\ g^{-1}$ Ni DW in the shoots (Reeves and Brooks, 1983); and (2) *Alyssum bertolonii* Desv., with up to 12,000 $\mu g\ g^{-1}$ Ni DW (Minguzzi and Vergnano Gambi, 1948).

In a preliminary survey of 65 plant *taxa* in NW Italy, only the Mediterranean shrub *Alyssoides utriculata* (L.) Medik. revealed variable Ni content in leaves (36–2236 $\mu g\ g^{-1}$ DW), suggesting this species as a possible Ni facultative hyperaccumulator (Roccotiello et al., 2010). Because this plant is an evergreen shrub, has a good biomass and has never been reported as a facultative hyperaccumulator (Reeves et al., 1983; Cecchi et al., 2010), the focus of our study was to conduct a rigorous and systematic test of Ni accumulation in field, in relation to soil chemistry, evaluating the potential for phytoremediation of *A. utriculata*. We collected plant specimens from serpentine and non-serpentine soils and analyzed both the soils and plants for various elements, including Ca, Mg, and Ni to examine potential correlation between bioavailable soil elements, plant uptake and translocation of those elements. We also evaluated the elements bound to root surfaces to assess possible phytostabilization (potential to immobilize metals at root level).

2. Materials and methods

Alyssoides utriculata (Brassicaceae) ranges primarily in the northeastern Mediterranean region. In Italy, the species grows on limestone, marble, sandstone, and serpentine where it is often found on rock outcrops, cliffs, and scree (Pignatti, 1982). *A. utriculata* is found on 30% of the serpentine areas in the Piedmont and Liguria regions of Italy (Marsili, 2010).

2.1. Sampling sites and sample collection

Plants were collected from two areas in NW Italy, containing serpentine and non-serpentine soils. The first area was at the Libiola sulfide mine (N 44°18′17″; E 9°26′57″), with two serpentine and one non-serpentine sampling sites. The serpentine sites were: (S1) on the toeslope of a spoil pile (tips), where the mineralogical composition is mainly represented by serpentinites ($\geqslant$50%), basalts and sulfide products, with a substrate showing a clear serpentinic trait (Marsili et al., 2009a; Marescotti et al., 2010); (S2) further mixed with soils derived from serpentine bedrock (Roccotiello et al., 2010). The non-serpentine site (NS) was located on a mine site, on soils derived from sandstone and shale bedrock (Marescotti et al., 2008).

The second area was in the eastern Ligurian Alps (Voltri Massif; N 44°28′49″, E 8°40′44″) and comprised one serpentine site (S3). The bedrock consisted of high-pressure meta-ophiolite like serpentines and metagabbros (Chiesa et al., 1975; Vanossi et al., 1984; Marsili et al., 2009b).

Five shoots from non-flowering branches and roots replicates (1 plant = 1 replicate) and five surrounding soil replicates (10–15 cm depth) were collected in Autumn (vegetative stage) from the three serpentine sites ($n = 5^*3 = 15$) and four shoot from non-flowering branches and root replicates and surrounding soil replicates were collected from the non-serpentine site ($n = 4$).

Soil pH was measured *in situ* using a portable pH meter (WTW PH330i, WTW, Munich, Germany) equipped with a glass electrode. Three pH replicates were measured at each serpentine site (S1, S2, S3) and three pH replicates were measured in each of the plant sample locations of the non-serpentine site (NS).

2.2. Plant and soil sample analysis

In the laboratory, plant samples were thoroughly rinsed first with tap water and then with deionized water to remove dust and soil particles. Leaves and roots were separated from stems. Stems were discarded based on previous analyses (Roccotiello et al., 2010; Roccotiello, 2011), showing an elemental content similar to that of roots, and because they are not used for the calculation of crucial parameters, such as translocation and bioaccumulation factors.

After oven drying (70 °C, 48 h), the leaves were weighed (DW) and before being powdered using a ball mill (Retsch MM2000, Haan, Germany), preceding the chemical analyses.

In order to evaluate whether significant binding of soil metals occurred on the roots (e.g. Prasad and Freitas, 2000), to assess *A. utriculata* possible use in phytostabilization, we performed an elution with ethylenediaminetetraacetic acid (EDTA) on the fresh thoroughly washed roots (only those from the serpentine sites). The washed roots were eluted in Na_2EDTA (20 mM) and shaken for 1.5 h at 150 rpm. This EDTA solution is the $Root_{EDTA}$ fraction. Subsequently, these roots were again rinsed in deionized water, to remove EDTA residues. All the roots, serpentine and non-serpentine, were then oven dried (70 °C, 48 h), weighed (DW) and powdered ($Root_{abs}$ and $Root_T$ fractions, respectively). The powdered roots and leaves (0.3 g DW) were acid digested in 65% HNO_3: 30% H_2O_2, 6:1.

Soil samples were oven-dried at 70 °C for 48 h before being sieved through a 2.0 mm mesh. Aliquots of dried, sieved soil were mixed with Na_2EDTA (1:40 w/v, 20 mM, pH 4.5) and agitated at 150 rpm for 3 h to extract the bioavailable metal fraction; the extract was acidified (1% HNO_3, v/v) before analyses.

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Soil and plant fractions were analyzed for Ca, Mg, and Ni concentration using Atomic Absorption Spectrometry (AAS) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The accuracy of the results was checked processing BCR-100 'beech leaves' reference material (JRC-IRMM, 2004). Plant and soil metal concentrations were expressed on a dry weight basis (DW). The total elemental content of the root, comprising both $\text{Root}_{\text{EDTA}}$ and Root_{abs}, was referred to as Root_{T} or simply 'Root;.

2.3. Data analysis

The mobility of nickel from soils into the roots and the ability to translocate the metals from the roots to the leaves were evaluated, respectively, by means of the bioaccumulation factor (BF) i.e. the Ni concentration ratio between leaf and soil, on a dry weight basis (Baker, 1981; Branquinho et al., 2007), and the translocation factor (TF) i.e. the Ni concentration ratio between leaf and Root_{T}, on a dry weight basis (Macnair, 2003).

The statistical analyses were performed using Statistica 8.0 (Statsoft Inc.) and SPSS statistics (version 19.0.0; IBM) software. The averages were presented with their standard deviations (SD). The results below the detection limits were presented as zero, and used as such in the calculations. The correlations between variables were evaluated using the Spearman's correlation coefficient (*rho*), since most data had a non-normal distribution. The Independent-samples MannWhitney-U test was used to evaluate differences between serpentine and non-serpentine samples. Non-parametric tests were used to avoid data transformation. Significance was considered at the $P < 0.05$ level.

3. Results

Soils from the three serpentine sites had significantly higher pH, significantly higher concentrations of all elements analyzed (including Ca) and a significantly lower Ca/Mg molar ratio, than the non-serpentine soil (Table 1). The soil pH shows a significant positive correlation with the soil bioavailable Ni ($rho = 0.775$; $P = 0.041$; $n = 7$).

Serpentine plants had significantly lower leaf Ca and significantly higher leaf Mg concentrations than non-serpentine plants, resulting in a significantly lower leaf Ca/Mg molar ratio for serpentine plants than non-serpentine plants (Fig. 1). The Ni in the roots of plants from serpentine soils was distributed 20% at the root surface ($\text{Root}_{\text{EDTA}}$) and 80% internally ($\text{Root}_{\text{abs}}$) (Fig. 1). Leaves of plants growing on serpentine soils had an average Ni concentration of 1065 $\mu g\ g^{-1}$ (hyperaccumulator status), in contrast to an average concentration of 146 $\mu g\ g^{-1}$ (accumulator, but not a hyperaccumulator) in plants growing on non-serpentine soil.

The bioaccumulation and translocation factors did not change between plants from serpentine and non-serpentine soils. Only for Ca the differences were significant, with higher values for the non-serpentine samples. The Ni BF was always greater than 2.5 and the TF greater than 2.8 (Fig. 2).

Table 1
Serpentine (high Ni) and non-serpentine (low Ni) soil bioavailable element concentrations and pH (Mean ± SD). All values are significantly different ($P < 0.05$).

	Sites	
	Serpentine ($n = 3$)	Non-serpentine ($n = 4$)
pH	6.60 ± 0.15 ($n = 15$)	4.51 ± 0.21 ($n = 4$)
Ca ($\mu g\ g^{-1}$)	1193.43 ± 451.01	556.78 ± 47.53
Mg ($\mu g\ g^{-1}$)	465.16 ± 171.75	93.05 ± 27.44
Ni ($\mu g\ g^{-1}$)	155.46 ± 75.89	11.09 ± 8.02
Ca/Mg (mol)	1.63 ± 0.55	3.92 ± 1.33

Interestingly, leaf Ca uptake was not significantly correlated with the amount of soil Ca, but was positively correlated with soil Ca/Mg ratio ($rho = 0.591$; $P = 0.008$; $n = 19$) and negatively correlated with soil Ni ($rho = -0.528$; $P = 0.02$; $n = 19$). Leaf Ca and Ni concentrations exhibited a significant negative correlation (Table 2) that was not found for the root concentrations. A significant positive correlation was found between Mg and Ni, in both leaves and roots, which was reflected in a negative correlation with Ca/Mg molar ratio for the same plant organs (Table 2). Root and leaf Ni concentrations were significantly and positively correlated with soil Ni bioavailability (Table 2).

Because of the significant correlation between soil pH and soil bioavailable Ni, likewise, there was a resulting significant positive correlation between soil pH and root and leaf tissue Ni concentration (respectively, $rho = 0.937$ and $rho = 0.901$; $n = 7$).

4. Discussion

Up to date a relatively small number of Mediterranean plant species is being studied for phytoremediation purposes (e.g. *Alyssum bertolonii* Desv.; *Alyssum murale* Waldst. & Kit., *Alyssum lesbiacum* (Candargy) Rech.f., *Alyssum corsicum* Duby) (Robinson et al., 1997; Bani et al., 2007; Singer et al., 2007; Broadhurst et al., 2008; Bani et al., 2010). Most well-known hyperaccumulator species may be of limited use for the remediation of metal contaminated soils in the Mediterranean area (e.g. *Thlaspi caerulescens* J.Presl & C.Presl) because of their sensitivity to heat and drought (Vangronsveld et al., 2009). Drought resistance and heat tolerance are prerequisites for survival and good performance under the prevailing weather conditions in the Mediterranean area (Barceló et al., 2001). *Alyssoides utriculata* seems interesting for all these aspects and for being an evergreen shrub able to grow on different soils. The greater abundance of *Alyssoides utriculata* in the Ligurian serpentine areas as compared to adjacent non-serpentine areas, suggests that it has preadaptation tolerance traits that allows it to grow on low-competition serpentine soils. However, the species does still persist on low-competition non-serpentine soils according to mechanisms already described for plant adaptation and evolution on serpentine soils (O'Dell and Rajakaruna, 2011). Controlled studies (usually in culture solution) have shown that bioavailable Ni concentrations, similar to the bioavailable concentrations in serpentine soils, result in nickel toxicity or, at the very least, measurable reduction in plant biomass (Nagy and Proctor, 1997), therefore, a serpentine adapted plant seems a good candidate for phytoremediation of Ni contaminated sites.

The leaves of *A. utriculata* collected from serpentine soils had an average leaf Ni concentration above 1000 $\mu g\ g^{-1}$, and in non-serpentine soils, 39.7–366 $\mu g\ g^{-1}$, in comparison, typical serpentine non-hyperaccumulator plants have 10–100 $\mu g\ g^{-1}$ Ni, in their leaves (Reeves, 1992), indicating the ability of the species to accumulate high quantities of Ni, even in soils with a low metal level. Being a facultative hyperaccumulator, it is not strange that previous surveys on soils with low Ni, done with colorimetric field dimethylgyoxime test (DMG) (Charlot, 1964) by Cecchi et al. (2010) and Roccotiello et al. (2010) and with analytical methods (Reeves et al., 1983) gave inconclusive results about Ni hyperaccumulation. However, in the same survey on 65 plant *taxa* in NW Italy by Roccotiello et al. (2010) *Alyssoides utriculata* tested positive to the DMG test when growing on serpentine soils.

The ability of plants to tolerate and accumulate heavy metals is useful both for phytoextraction (i.e. soil cleaning via metal accumulation in plant shoots) and for phytostabilization (i.e. metals immobilization at the root level) purposes. Plants with both

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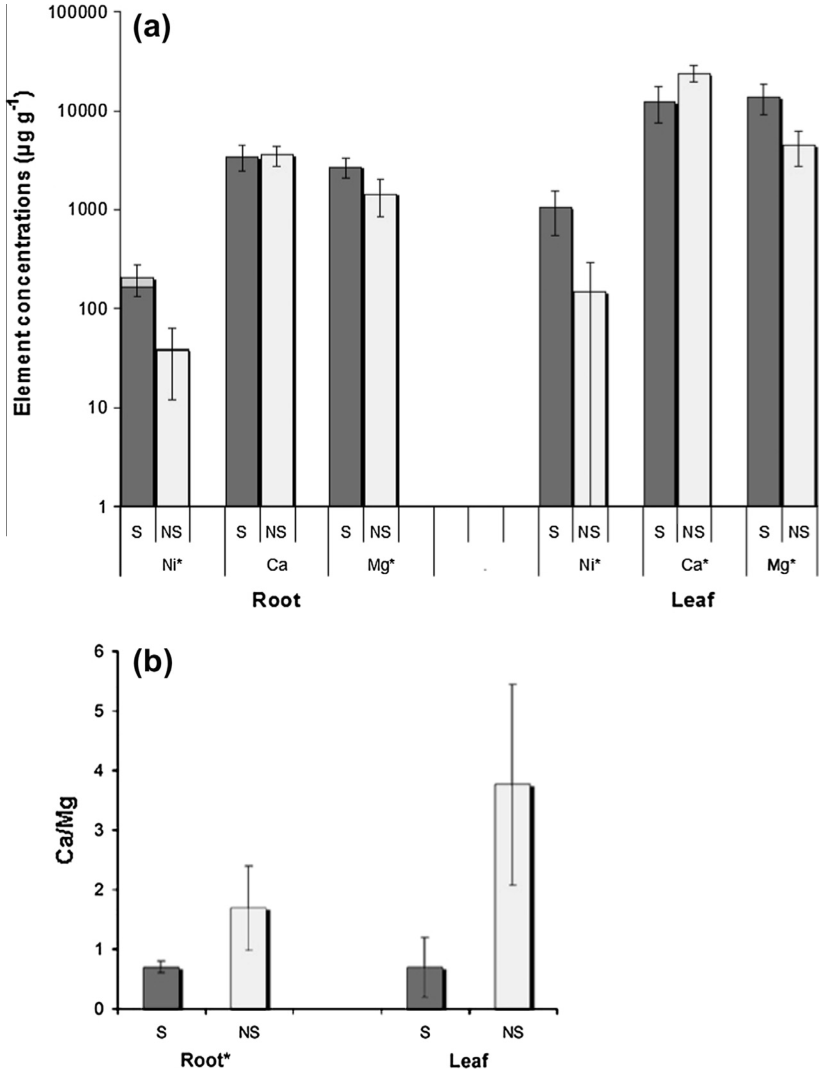

Fig. 1. Serpentine (S) and non-serpentine (NS) *Alyssoides utriculata* root and leaf (a) element concentrations and (b) Ca/Mg molar ratio. Means (serpentine $n = 15$; non-serpentine $n = 4$) ± SD; * = significant difference ($P < 0.05$) between serpentine and non-serpentine element concentrations. Data on Ni, Ca and Mg concentrations are expressed on a log scale. *Note:* in serpentine roots, the Ni column is divided between $\text{Root}_{\text{EDTA}}$ on top ($41.55 \pm 17.66\ \mu\text{g g}^{-1}$) and Root_{abs} on the bottom ($163.00 \pm 59.52\ \mu\text{g g}^{-1}$); all the other Root columns represent the total Root_{T}.

bioaccumulation factor and translocation factor greater than one (TF and BF > 1) have the potential to be used in phytoextraction (Baker and Whiting, 2002; Cai and Ma, 2003; McGrath and Zhao, 2003; Pilon-Smits, 2005). Besides, plants with bioaccumulation factor greater than one and translocation factor less than one (BF > 1 and TF < 1) have the potential for phytostabilization (Yoon et al., 2006). The Ni BFs of *A. utriculata* were between 2.5 and 20.3, depending on the soil bioavailable Ni, indicating its potential for phytoremediation in field conditions (Reeves and Baker, 2000). In addition, the TFs of Ni in *A. utriculata* ranged from 1.2 to 8.5, indicating that once Ni is present inside the root it is easily translocated to the shoot (Baker, 1981; Reeves and Baker, 2000). The strong nickel accumulation in the leaves, combined with high BFs and TFs, indicate that *A. utriculata* is potentially useful in the phytoextraction of nickel contaminated sites. Although 20% of the Ni concentration accounted for the root is adsorbed to external surfaces, this represents only about 3% of the total Ni concentration in the plant, as such, its accumulation abilities are far more interesting in this plant than those of rhizostabilization are. Most works that describe root metal concentrations wash the roots in water. Considering this, the final concentration given for that organ includes some percentage of metals not yet uptaken. The choice of EDTA to chelate any metals adsorbed to root was employed by several authors (Azcue, 1996; Branquinho et al., 1997; Prasad and Freitas, 2000; Branquinho et al., 2007; Serrano et al., 2011) to remove an amount of adsorbed ions greater than the traditional water wash; the remaining Ni in the root would be a better estimate of the Ni physiologically uptaken by the plant.

Knowing *A. utriculata* plants can reach a maximum height of 20–40 cm (Pignatti, 1982) with a diameter up to 100 cm (personal observation), but are slow growers (5–8 cm in height per year, estimation from personal observations), maybe they could be used as a first tool in the revegetation of Ni contaminated sites, providing protection to the soil while removing the excess Ni, stored in the harvestable leaves.

The differences in leaf Mg and Ni concentration, in *A. utriculata*, between serpentine and non-serpentine sites, primarily reflect soil nutrient bioavailability (Reeves and Baker, 1984; Westerbergh and Saura, 1992; Westerbergh, 1994; Boyd and Martens, 1998; Reeves, 2006; Ghasemi and Ghaderian, 2009). In the case of Ca however, *A. utriculata* showed an accumulation pattern related to the soil available Ca/Mg (positive) or Ni (negative), not to the soil available Ca.

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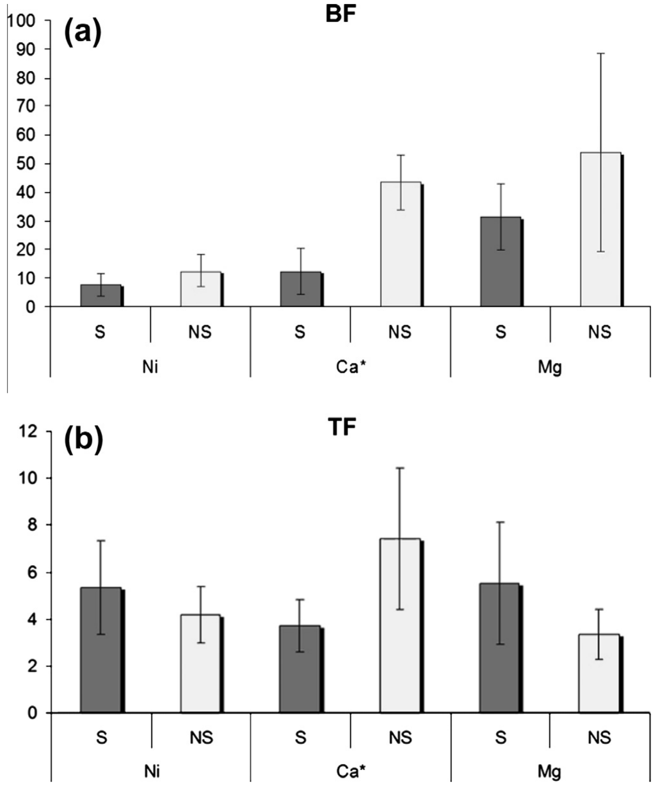

Fig. 2. (a) Bioaccumulation (BF) and (b) translocation factors (TF) for serpentine ($n = 15$) and non-serpentine ($n = 4$) plants. Means ± SD. * indicates significant differences between serpentine and non-serpentine samples.

Table 2
Significant Spearman's rank correlation coefficients between Ni and Ca, Mg and Ca/Mg for soil ($n = 19$); roots ($n = 19$) and leaves ($n = 19$); and the bioaccumulation (BF, $n = 19$) and translocation factors (TF, $n = 19$). ns = Not significant ($P \geqslant 0.05$).

Parameter	Element	Ni			
		Root	Leaf	BF	TF
	Ca	ns	−0.644	ns	ns
	Mg	0.696	0.684	0.496	0.742
	Ca/Mg	−0.582	−0.758	ns	−0.530
Soil	Ni	0.581	0.746	−0.537	ns
Root	Ni	–	0.877	ns	ns
Leaf	Ni	0.877	–	ns	0.561

Mg and Ca may be competing for uptake by *A. utriculata* and due to the high availability of Ni, the uptake of Mg is favored. Brooks et al. (1981) noted that the various subspecies of *Alyssum serpyllifolium* decreased their uptake of Ca, and increased that of Mg, in the presence of high concentrations of Ni. Some studies have also shown the reduction of the leaf Ni when the leaf Ca/Mg is higher (Proctor and McGowan, 1976; Robertson, 1985, 1992; Parker et al., 1998) and our results seem to be comparable.

It is noticeable that none of the chemical extraction methods like $CaCl_2$, DTPA, ammonium acetate (van Raij, 1998) and EDTA (Branquinho et al., 1997, 2007; Serrano et al. 2011) employed to evaluate the soil available metal fraction has yet, universally and accurately, replicated the real bioavailable fraction for hyperaccumulators (Van der Ent et al., 2013). The EDTA-soil-fraction represents the "easily bioavailable" fraction and not the "immediately bioavailable" one as already observed by D'Amico (2009). Nevertheless, this fraction produced good correlations with plant metal contents, specifically Ni, which is the main reason for using that estimate instead of total soil concentrations (Ure et al., 1993; Wang et al., 2004; Roccotiello et al., 2010). It is always difficult to establish cause-effect relationships based on correlations between chemical elements using only data from field conditions. However, patterns obtained under natural field conditions are the genuine ones, and are thus important to disclose.

5. Conclusion

Considering the results of this study, and in accordance to Reeves and Baker (2000) and Van der Ent et al. (2013), we can confirm *A. utriculata* as a Ni hyperaccumulator plant. Specifically, *A. utriculata* can be classified as a facultative hyperaccumulator species (Reeves, 2006), since plants only attained leaf Ni concentrations >1000 $\mu g\,g^{-1}$ when grown on Ni-rich soil (serpentine) in contrast to plants growing on non-serpentine soils which only attained a maximum leaf Ni concentration of <500 $\mu g\,g^{-1}$.

This hyperaccumulation trait, together with BF > 1 and TF > 1 support the evidence of the phytoremediation potential for this species. Nonetheless, for a definitive classification of *A. utriculata* as a really Ni phytoremediator further investigations are required to ascertain the efficiency of the phytoextraction, namely growing plants from seed in a controlled environment.

The ability of *A. utriculata* to grow on both serpentine and non-serpentine substrates conveys a great opportunity for the species to explore a wider diversity of habitat types and to maintain large, viable populations in Ni-contaminated soils of the Mediterranean area.

6. Uncited references

Bencko (1983) and McGrath et al. (1999).

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