



Diversity of nickel response in Tuscan accessions of the facultative serpentinophyte *Silene paradoxa* L

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Abstract

Aims The aim of this work was to shed light on the still debated mechanisms of Ni tolerance in facultative serpentinophytes and their photosynthetic performance in presence of Ni. To this end, three non-serpentine and three serpentine accessions of *Silene paradoxa* L. collected in Tuscany were compared.

Methods Plants were grown in hydroponics with NiSO₄ treatments and root elongation, Ni accumulation, and photosynthetic performance evaluated.

Results The presence of Ni reduced plant growth and led to different metal accumulation patterns

between the two plant groups. Tolerance and accumulation parameters suggest that serpentine adaptation required enhanced Ni tolerance in *S. paradoxa*, likely driven by the selective pressure imposed by high metal concentration in the substrate. Ni tolerance was linked to the ability to limit the metal entry in the root symplast rather than being associated with high tissue tolerance or shoot exclusion. Leaf gas exchange data and chlorophyll fluorescence data revealed that Ni excess differentially affected stomatal opening in the two plant groups, with cascading effects on assimilation rates and, consequently, growth. Ni excess, rather than affecting leaf photochemistry per se, differentially affected stomatal opening, with declines in stomatal conductance observed only in the non-serpentine accession. This indicates that greater Ni tolerance in the serpentine accession was linked with improved water relations rather than enhanced Ni tissue tolerance at the photosynthetic level.

Conclusions Overall, all results indicate that metal exclusion is the main mechanism of Ni tolerance in *S. paradoxa*, thus reducing the need for more energy-demanding strategies of internal tolerance.

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Introduction

Although life cannot exist without the contribution of metal ions, when present above a certain threshold, trace elements represent environmental pollutants of concern for living organisms (Nagajyoti et al. 2010). Different metals accumulate in soils, with lead, chromium, zinc, cadmium, nickel and copper being the most common in contaminated sites (USEPA, 1996). Their accumulation in soil can have geogenic (i.e. mainly due to weathering of metal-rich parent materials or rock-outcropping) and anthropogenic origins (i.e., from human activities such as mining and smelting operations, and agriculture, Madhav et al., 2024). The toxicity threshold for plants varies depending on the element physico-chemical properties and is influenced by less predictable factors, such as plant species, chemical form, soil composition, and pH (Nagajyoti et al. 2010). Excess trace metals, however, invariably have severe consequences for plant life and biodiversity (Nawaz et al. 2023). Despite the diverse toxicity mechanisms exerted by trace metals on plants (Tang et al. 2023), "metallophytes" have evolved remarkable adaptations that enabled them to survive and reproduce in metal-enriched soils (Wójcik et al. 2017).

These species have evolved particular traits to cope with the surplus of essential or non-essential elements (Ernst 2006). They can either restrict metal entry in the root and/or its transport to the shoot (and thus termed "excluders", Baker 1981) or, more rarely, tolerate metals by accumulating them in their shoots at extraordinarily high concentrations (i.e. "accumulating" and "hyperaccumulating" species, van der Ent et al. 2013). Regardless of their strategy, metallophytes claim priorities for conservation of their biodiversity as they are excellent biological systems to study plant adaptation to extreme environments. Furthermore, they are also the optimal choice for the development of environmental technologies for the remediation of metal-contaminated soils (Whiting et al. 2004).

Among the metallophytes, "serpentinophytes" are adapted to the harsh conditions of serpentine soils (Wójcik et al. 2017), considered among the most challenging settings for plant life (Konečná et al. 2020). Representing a peculiar blend of stressors, serpentine substrates are mainly characterised by abundant toxic metals (e.g. Ni, Co and Cr), Mg excess, low nutrient

contents (in particular Ca), low water retention and a rocky structure that affects root development (Brady et al. 2005; Brooks 1987; Gonnelli and Renella 2012). Among all of these stressors, excessive Ni is considered to be one of the most critical factors contributing to serpentine toxicity (Brooks 1987) which, coupled with the unfavourable Mg:Ca ratio, drive the adaptive responses to the "serpentine syndrome" (Brady et al. 2005).

Due to their adaptations, serpentinophytes have long fascinated biologists, providing key insights into ecological specialization through physiological changes (Konečná et al. 2020). Most research has so far focused on Ni hyperaccumulating serpentinophytes, not only because metal accumulation is a fascinating topic per se but also for their practical application in phytoremediation and agromining techniques (Kidd et al. 2018). As a result, several mechanisms underlying Ni hyperaccumulation have been described, such as the positive relationship between Ni tolerance and accumulation (Galardi et al. 2007; Bettarini et al. 2021; Colzi et al. 2023), and the molecular mechanisms underlying this trait (van der Pas and Ingle 2019), and the impact of Ni on photosynthetic physiology (Scartazza et al. 2022; Colzi et al. 2023).

Far less is known about the physiological mechanisms that allow excluding plants to adapt to excessive Ni concentrations in serpentine soils, with contrasting information in the literature on the relationship between Ni exclusion and tolerance. For example, in *Silene dioica*, accessions from serpentine and non-serpentine substrates had similar Ni tolerance in hydroponics (Westerbergh 1994), whereas in *Knautia arvensis* and *Arabidopsis lyrata* serpentine plants had higher Ni tolerance and lower Ni accumulation compared to non-serpentine ones (Kolář et al. 2014a; Veatch-Blohm et al. 2017). Higher tolerance in serpentine accessions was also found in *Caulanthus amplexicaulis* and *Pistacia atlantica* (Burrell et al. 2012; Pakdaman et al. 2013), but without significant differences in Ni accumulation. By contrast, higher Ni accumulation was found in a serpentine accession of *S. vulgaris* compared to a less Ni-tolerant non-serpentine accession (Koszelnik-Leszek and Bielecki 2013). These findings highlight the very heterogeneous and species-specific nature of the relationship between tolerance and accumulation in excluders, which is conceivable given the biodiversity found in nature.

Nevertheless, definite conclusions on the mechanisms underlying high Ni tolerance cannot be drawn as most studies were either based on: i) only two accessions selected arbitrarily from soils with contrasting features, ii) a comparison of closely related species and not on facultative serpentinophytes sensu *strictu*, iii) only on root or shoot accumulation data, iv) undesorbed roots, or v) too high concentrations to compare plants in a functioning physiological state.

On the other hand, a totally unexplored issue is the impact of Ni on photosynthesis in excluding serpentinophytes. Photosynthetic performance is a key process whose protection from Ni toxicity can be directly linked to growth capacity on serpentine substrates, yet studies on the effect of Ni on photosynthesis have only focused on non-serpentinophytes or Ni hyperaccumulators. In non-serpentinophytes, Ni disrupts different components involved in photosynthetic performance (Seregin and Kozhevnikova 2006; Sreekanth et al. 2013), reducing electron transport rates (Mohanty et al. 1989; Krupa and Baszynski 1995), inhibiting Calvin cycle enzymes (Sheoran et al. 1990), and decreasing both stomatal conductance (Sheoran et al. 1990; Bishnoi et al. 1993) and mesophyll CO_2 diffusion rates (Velikova et al. 2011). By contrast, in the Ni-hyperaccumulating *Odontarrhena* species, Ni improved both the biophysics and biochemistry of photosynthesis, with toxic effects only at very high metal concentrations (Scartazza et al. 2022).

To address the critical question of whether, and how, Ni accumulation is linked with tolerance in excluding facultative metallophytes, we compared metal response in three serpentine and three non-serpentine accessions of *S. paradoxa* L. (Caryophyllaceae). This species, widespread in Southern Europe, is considered as an excluder type and is an excellent model system to study the evolution and the eco-physiology of trace metal tolerance, with several accessions adapted to grow on serpentine soils and on mine deposits (Pignatti 1997; Chiarucci et al. 1995; Mengoni et al. 2000, 2001). Given its wide diffusion on diverse metalliferous substrates, we hypothesize that serpentine accessions of *S. paradoxa* exhibit enhanced Ni tolerance mainly through root exclusion rather than internal detoxification mechanisms. Furthermore, to explore further the overlooked issue of Ni excess in the substrate on photosynthesis in excluding serpentinophytes, we

assessed the photosynthetic performance in presence of Ni in contrasting and *ad-hoc* selected pair of accessions. This comparison enables us to test our second hypothesis, that the serpentine accession maintained photosynthetic functions with high root-zone Ni by limiting Ni entry into the plant, without an increase in Ni tolerance in photosynthetic tissues.

Materials and methods

Plant and soil material and collection

Plant material and seeds of *Silene paradoxa* L. were collected from native accessions in July 2022 in three distant serpentine outcrops and in three control sites, situated in Tuscany (Italy), information about localities is given in Table 1.

At each site, shoots from five well developed 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. In addition, 10 bulk soil samples (ca. 20 g) were collected at ca. 5–15 cm depth in the same sampling area. These samples were then pooled together to obtain a single bulk soil sample of ca. 200 g.

Nickel concentrations in field-collected samples

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 subsamples (0.5 g d.w.) were digested using 10 mL of 69% HNO_3 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. Shoot samples were carefully washed three times with deionized water, dried at 50 °C for 48 h (Selvi et al. 2017), and then the dried material (0.1 g d.w.) was analyzed as described for soils. Each plant analysis was replicated three times.

Table 1 List of seed collection sites of *S. paradoxa*, with accession code, coordinates, altitude and substrate. Nickel concentrations in the native soil ($\mu\text{g g}^{-1}$ d.w., mean of 5 val-ues $\pm$ standard deviation, total concentration) and in shoots of field collected plants are given ($\mu\text{g g}^{-1}$ d.w., mean of 5 values $\pm$ standard deviation)

Accession	Code	Lat, Long	Altitude (m)	Substrate	[Ni] soil($\mu\text{g g}^{-1}$ d.w.)	[Ni] shoot($\mu\text{g g}^{-1}$ d.w.)
Non serpentine (NS)						
Monte Acuto	Ma	43°03'27'', 11°18'50''	430 – 440	Marl	37 $\pm$ 5	2.3 $\pm$ 0.8
Monte Ceceri	Mc	43°48'09'', 11°18'34''	280 – 300	Marl and sandstone	88 $\pm$ 12	4.1 $\pm$ 0.9
Colle Val d'Elsa	Co	43°24'0.5'', 11°07'49''	170 – 180	Calcareous	79 $\pm$ 8	1.9 $\pm$ 0.5
Serpentine (S)						
Pieve S. Stefano	Pi	43°38'57'', 12°03'22''	450 – 480	Serpentine	1412 $\pm$ 71	17.3 $\pm$ 1.2
Impruneta	Im	43°40'37'', 11°16'07''	270 – 280	Serpentine	1089 $\pm$ 83	12.2 $\pm$ 0.9
Pomaia	Po	43°21'46'', 10°34'59''	130 – 140	Serpentine	1516 $\pm$ 146	14.2 $\pm$ 1.4

Experiment 1

Nickel tolerance and accumulation in hydroponics

Seeds were sown in peat soil and placed in a growth chamber (24/16 °C day/night; light intensity $300 \mu\text{mol m}^{-2} \text{s}^{-1}$, 16-h day photoperiod; relative humidity 60–65%). After 10 days, seedlings were transferred to hydroponic cultures in 1-L polyethylene pots (one plant per pot) containing a modified half-strength Hoagland' 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 (*cf.* Bettarini et al. 2020). Nutrient solutions were changed weekly, and plants were grown in the same growth chamber described above. After three weeks, plants of homogeneous size (12 plants per treatment) were selected and treated as in Bettarini et al. (2021): the length of the longest root of each plant was measured with a ruler and subsequently plants were exposed for seven days to a series of NiSO_4 concentrations (0, 20, 40, 60, 100, 150 μM), in a background solution of the same composition as the pre-culture solution. Metal concentrations were in the same order of magnitude, or one higher, to Ni concentrations found in soil solutions of serpentine substrates (Proctor et al. 1981; Ly et al. 2024). 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). Metal-imposed growth inhibition was evaluated in roots since it has been demonstrated, including in plants from the genus *Silene*, that it is completely attributable to toxic metal accumulation in the root itself, independently of shoot responses (De Knecht et al. 1992; Harmens et al. 1993; van der Ent et al. 2024). Plants were rinsed with milliQ-water and the roots of six plants per treatment were carefully washed with 10 mM $\text{Pb}(\text{NO}_3)_2$ at 4°C for 30 min to desorb metals adhering to the root cell wall, as in Bazihizina et al. (2015). Shoots and roots were dried at 70 °C for 24 h and Ni accumulation was determined as previously stated.

The experimental data points were fitted with the four-parameter log-logistic function for the analysis of the growth and accumulation response to Ni treatment. Root length increment or root Ni concentration were measured as response variable for all tested accessions, while the Ni concentration of the growth medium was considered as the predictor variable. Data fitting to dose–response models allowed to estimate the half-maximal effects of both external and internal Ni concentrations ($\text{EC}_{50_{\text{ext}}}$ and $\text{EC}_{50_{\text{int}}}$, respectively). The drc package (Ritz et al., 2015) implemented in R Studio version R 3.4.3 (R Core Team 2017) was used to fit the curve of concentration–response data. Furthermore, after obtaining the linear correlation between Ni concentration in roots and shoots, we used the angular coefficient of the obtained regression line as a descriptor of the shoot:root metal concentration ratio (translocation factor), as in Galardi et al. (2007).

Experiment 2

Plant growth and Ni accumulation

For the experiment focusing on Ni impact on the photosynthetic process, based on the results obtained in Experiment 1 we selected the non-serpentine Co and the serpentine Pi accessions. This selection was made considering that among the various mechanisms of metal exclusion (see for example Dal Corso et al. 2013; Wójcik et al. 2017), hydroponic experiments with root desorption exclude those operating outside the symplast, e.g. association with rhizosphere microorganisms and metal sequestration in the rhizosphere and/or in the cell wall. In particular, we based our selection on the internal metal concentration, that can result from lower membrane permeability and/or active efflux and represents the amount of metal to be detoxified through cytosolic chelation and/or vacuole sequestration (as explained in section “Growth and Ni accumulation over time”).

Plants were cultivated as above mentioned, but with only the three lowest NiSO_4 concentrations (20, 40 and 60 μM) and the treatment lasted for four weeks, with plants (12 plants per treatment) harvested at different time points (0, 7, 17 and 27 days after treatment). Nutrient solutions were renewed weekly and, for each harvest, plant growth and shoot and root Ni concentration were measured as above described. In addition, time-dependant changes in photosynthetic parameters were measured and final chlorophyll concentration and stomatal density and size assessed as described below.

Experimental data of Ni accumulation in roots and shoots or plant growth over time were analysed by linear regression (using GraphPad Prism 7, GraphPad Software, San Diego, CA). The angular coefficient (slope) of the linear regression was assumed as a descriptor of the translocation factor or of the plant growth rate, according to the experiment.

Gas exchange analysis and chlorophyll fluorescence measurements

Leaf gas exchange and chlorophyll a fluorescence parameters were measured prior to each harvest (at 0, 7, 17 and 27 days after treatment) using a portable gas exchange system (LI-6400XT; Li-Cor Inc., Lincoln, NE) equipped with an integrated fluorescence

chamber head (LI-6400-40; Li-Cor Inc.). At all time points, measurements were taken from 9:00 to 13:00 as in Bazihizina et al. (2015) with reference CO_2 of 400 $\mu\text{mol mol}^{-1}$, ambient relative humidity, flow rate of 500 $\mu\text{mol s}^{-1}$, chamber temperature at 20 °C and 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation. Measurements were taken on a youngest fully expanded leaf in six plants per treatment and the following leaf gas exchange and fluorescence parameters measured (Maxwell and Johnson 2000): net photosynthetic rate (P_n), stomatal conductance (g_s), internal CO_2 concentrations (C_i), the effective PSII quantum yield (ΦPSII); photochemical quenching (qP); the non-photochemical quenching (qN); and the electron transport rate (ETR).

Stomatal density and stomatal size

Stomatal density was assessed using the nail polish method on young fully expanded leaves (Ceulemans et al. 1995). Leaf imprints were then examined with a Zeiss Axio Observer Z1 fluorescence microscope (Carl Zeiss, Thornwood, NY) and five areas of 0.361 mm^2 of the same leaf observed for the stomatal count. Measurements of stomatal size (12 stomata per leaf) were performed on three different leaves of the same plant. Stomatal density and size were measured manually using the Adobe Photoshop 7.0 software.

Data analyses

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

The data of plant length increment and accumulation over time were analysed fitting linear mixed-effects models (LMMs) in a repeated measurement ANOVA design, using treatment as a random effect factor to account for the temporal correlation of observations. Time was used as an ordinal variable. The physiological effects of the treatment were investigated using plant length increment and accumulation values as response variables and the treatments as explanatory variables in a full factorial design. ANOVA type III table, with Satterthwaite’s method was used to verify the significance of the fixed effects

and of associated interaction factors. The analyses were run with the free software R. LMM computations were performed using the lmer function of the lme4 package version 1.1–12 for fitting the models.

Results

Experiment 1

Nickel tolerance and accumulation in serpentine and non-serpentine accessions

Root length increments in *S. paradoxa* accessions exposed to increasing NiSO_4 concentrations for seven days are shown in Fig. 1. In the low-dose zone, NS plants already showed a significant decrease in root growth compared to the respective controls, while S plants were generally not affected, or even displayed an enhanced, but not significant, growth (Im and, especially, Pi accessions). At the highest metal levels, there was a general reduction in root elongation in all accessions. Two-way ANOVA showed significant interpopulation variation in root elongation in response to Ni treatments (Table S1) and a significant interaction accession*treatment. Data modelling gave significant fitting for four-parameter dose–response curves, with p-values in the range of 0.0001–0.045 (Table 2), thus validating

the model for all examined accessions. Significant variation in $\text{EC}_{50_{\text{ext}}}$ was found among the accessions (Table S2a), with the NS plants equally exhibiting the lowest values and the S ones the highest, with Pi accession identified as the most tolerant to external Ni concentration.

In all accessions, Ni concentration in roots and shoots increased with increasing external NiSO_4 . Roots showed values higher than shoots in almost all treatments (Fig. 2). However, significant differences were observed amongst accessions in both root and shoot Ni accumulation. Two-way ANOVA indicated significant variation among accessions and a significant effect accession*treatment for both root and shoot metal concentrations (Table S3). In particular, while Ni accumulation in the roots of S plants remained relatively consistent, shoots accumulation trends varied, with Pi and Im accessions showing the lowest values for most of the concentrations used. Root accumulation data were used to calculate the tolerance parameters based also on internal Ni concentration ($\text{EC}_{50_{\text{int}}}$, $0.036 < p < 0.0487$, Tables 2). Values of $\text{EC}_{50_{\text{int}}}$ were different among accessions (Table S2b), with Ma plants identified as the most sensitive to internal high Ni concentration and Po plants as the most tolerant. In any case, differences in $\text{EC}_{50_{\text{int}}}$ values were not related to substrate of origin, as the mean value in the S accessions was higher than in the NS but not significantly.

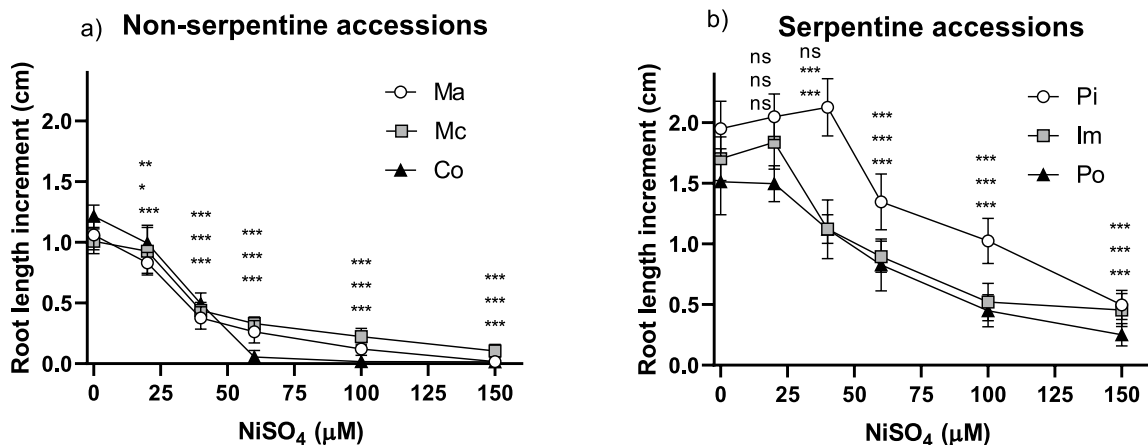


Fig. 1 Increment in root length (cm) in non-serpentine and serpentine *S. paradoxa* accessions treated with increasing NiSO_4 concentrations for seven days. a) root length of non-serpentine accessions; b) root length of serpentine accessions. Asterisks indicate the significant differences in root length

within each accession compared to its control according to the Dunnett's test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (the sequence of the indications reflects the one of the legends: Ma, Mc, Co and Pi, Im, Po respectively). Values are means of 12 replicates $\pm$ standard deviation

Table 2 $EC_{50_{ext}}$ and $EC_{50_{int}}$ of the root dose–response curves with the four parameters model for six *S. paradoxa* accessions grown at five $NiSO_4$ concentrations. Letters indicate significant differences among accessions according to the Tukey's test

Accession	P value	$EC_{50_{ext}}(\mu M)$		P value	$EC_{50_{int}}(\mu g\ g^{-1}\ d.w.)$		
Non-serpentine							
Ma	0.0103	32.5	±	1.7 a	0.0098	208.3	± 13.5 a
Mc	0.0330	34.3	±	1.9 a	0.0421	272.1	± 22.4 b
Co	0.0003	34.8	±	1.3 a	0.0179	289.8	± 15.3 b
Mean ± sd		33.9	±	1.2		256.7	± 42.9
Serpentine							
Pi	0.0004	78.5	±	10.7 d	0.0072	271.2	± 18.2 b
Im	0.0001	43.9	±	2.6 b	0.0036	293.3	± 20.5 b
Po	0.045	58.6	±	6.2 c	0.0487	402.2	± 81.7 c
Mean ± sd		60.3	±	17.4 *		322.2	± 70.2

($p < 0.05$). Asterisks indicate significant differences between the mean values of the non-serpentine and serpentine accessions (* $p < 0.05$, ** $p < 0.01$)

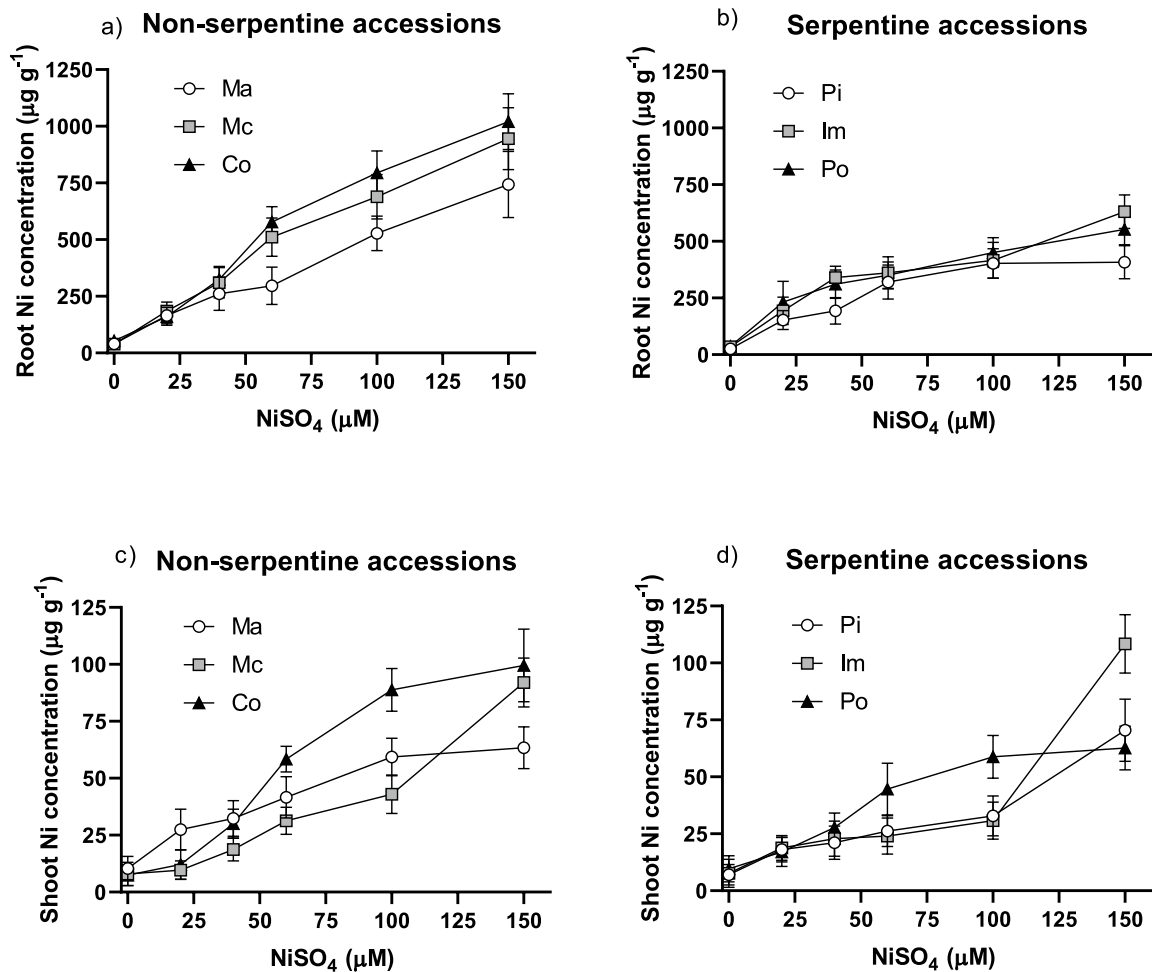


Fig. 2 Nickel accumulation ($\mu g\ g^{-1}\ d.w.$) in roots and shoots of the non-serpentine and serpentine *S. paradoxa* accessions treated with five $NiSO_4$ concentrations for seven days. a) root Ni concentration in non-serpentine accessions; b) root Ni con-

centration in serpentine accessions; c) shoot Ni concentration in non-serpentine accessions; d) shoot Ni concentration in serpentine accessions. Values are means of 12 replicates $\pm$ standard deviation

Each accession showed a significant positive linear correlation between Ni concentration in roots and shoots ($0.9085 < R < 0.9878$, $p < 0.0001$, Table 3). The angular coefficient of this regression line, used as a proxy of the translocation factor, showed significant variation among accessions (Table S4), but similar mean values between the S and the NS plants (Table 3).

Experiment 2

Growth and Ni accumulation over time

As above mentioned, based on the results obtained in Experiment 1, we selected Co and Pi accessions to evaluate the effect of Ni at the photosynthesis level. This because these two contrasting accessions had different $EC50_{ext}$ (with the Pi plants as the most tolerant among all the S plants) despite having comparable $EC50_{int}$ and translocation coefficients. These data thus suggest that both accessions had a similar sensitivity to Ni at the tissue level and similar exclusion from the shoots. Therefore, given the superior tolerance compared to Co plants, we hypothesized that Pi plants were likely to rely mainly on mechanisms that

limit metal entry at the root level rather than on internal Ni detoxification at the cellular level.

For a correct data interpretation, before assessing the time-dependent effect of Ni on photosynthetic parameters, we evaluated growth and accumulation in the same experimental conditions. Furthermore, to work in realistic conditions where the plants are alive and allow the measurement of reliable and physiologically relevant data (van der Ent et al. 2024), samples were exposed to the lowest Ni concentrations (i.e. 20, 40 and 60 μM NiSO_4) and not the full series of concentrations used in experiment 1, where very high concentrations were needed to reach growth values approaching zero for significant data modelling and calculation of tolerance parameters.

At each sampling time, one-way ANOVA highlighted significant differences in root elongation among the treatments (Fig. 3, $95.33 < F < 310.2$, $p < 0.0001$ and $31.00 < F < 62.28$, $p < 0.0001$ for all treatment times, for the NS and the S accession, respectively). While in controls there was a progressive increment in root length over time, Ni treatment significantly reduced growth in NS samples. In S samples, the presence of Ni in the culture medium generally increased root length increment compared to controls at the two lowest concentrations used, while only the highest concentration reduced it. Root elongation changed significantly depending on the treatment (interaction treatment*time significant with $p < 0.001$, Table S5) and, for a more direct comparison among the samples, elongation rate was estimated as the angular coefficient (slope) of the regression line between increment in length and sampling time, as in Falsini et al (2022). Compared to control conditions, in NS plants the growth rate significantly decreased following the increase of Ni concentration in the substrate. By contrast, in S plants it significantly increased at the lowest Ni concentrations, peaking with 20 μM NiSO_4 , and then progressively decreased with further increases of metal concentrations in the substrate, reaching control values with 60 μM NiSO_4 (Table 4a). This coefficient showed significant differences between accessions (Table S6).

Nickel concentrations in roots and shoots of both accessions increased with increasing metal concentrations in the root-zone for all NiSO_4 concentrations used (Fig. 4). Nickel levels in shoots were much lower than those in roots for both accessions. Statistically significant differences in root and shoot

Table 3 Shoot:root ratio in nickel concentration of six *S. paradoxa* accessions, calculated as the angular coefficient of the linear regression between Ni shoot concentration and Ni root concentration in plants treated for seven days with NiSO_4 . Values are means of 12 replicates $\pm$ standard error. Letters indicate significant differences among accessions according to the Tukey's test ($p < 0.05$). Asterisks indicate significant differences between the mean values of the non-serpentine and serpentine accessions (* $p < 0.05$, ** $p < 0.01$, ns = non significant)

Accession	p value	Slope		R
Non-serpentine				
Ma	<0.0001	0.103 $\pm$	0.01 ab	0.9615
Mc	<0.0001	0.080 $\pm$	0.003 a	0.9686
Co	<0.0001	0.102 $\pm$	0.002 ab	0.9878
Mean $\pm$ sd		0.095 $\pm$	0.013	
Serpentine				
Pi	<0.0001	0.116 $\pm$	0.006 b	0.9284
Im	<0.0001	0.118 $\pm$	0.007 b	0.9085
Po	<0.0001	0.114 $\pm$	0.004 b	0.9702
Mean $\pm$ sd		0.116 $\pm$	0.002 ns	

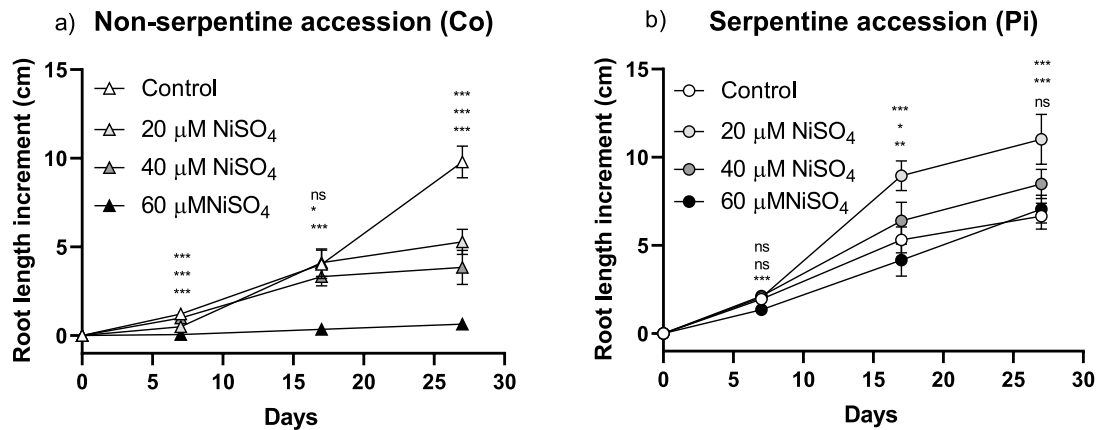


Fig. 3 Increment in length (cm) of NS (Co) and S (Pi) accessions of *S. paradoxa* treated with increasing NiSO_4 for 27 days. Increment was calculated subtracting length values at the beginning of the treatment to values at the end of the

Ni concentrations were found between the two accessions at almost all NiSO_4 concentrations, with a significant interaction treatment*time (Table S7). Generally, S plants had the lowest values; only in the case of $60 \mu\text{M NiSO}_4$ treatment, the accumulation values were similar between accessions, especially for shoots.

The calculation of the shoot:root Ni concentration ratio, as the coefficient of the regression line, gave significant results for both the accessions at all the metal treatments (Table 4b). Values were below unit and significant variation was found among them (Table S8), with an increasing trend in both the accessions. The shoot:root Ni concentration ratios were similar between S and NS plants, with the only exception at $40 \mu\text{M NiSO}_4$ where the S accession had higher values than the NS one.

Photosynthetic parameters

Gas exchange measurements highlighted a different behaviour in the two accessions in response to the highest Ni concentration in the substrate compared to controls. While in Ni-treated NS plants there was a significant decline for P_n , g_s , and C_i (82%, 95% and 52% reduction compared to controls, respectively, Fig. 5 a, c, e), in Ni-treated S plants this did not occur (Fig. 5 b, d, f). Indeed, in S plants, the presence of Ni

treatment. Asterisks above the data points of the 12 replicates indicate the significant differences compared to the control at each single exposure time according to the Dunnett's test (at least $p < 0.05$)

in the substrate generally led to greater P_n , g_s and C_i compared controls (Fig. 5 b, d, f).

As for gas exchange data, time-dependent changes in chlorophyll a fluorescence parameter in light-adapted leaves highlighted a different trend between the two accessions, particularly for the highest Ni concentration (Fig. 6). In NS plants, up to $40 \mu\text{M NiSO}_4$, in general there were no significant difference for the measured chlorophyll fluorescence parameters, except for qN which declined compared to control plants. By contrast, at $60 \mu\text{M NiSO}_4$, ΦPSII , ETR and qN declined compared to control plants from seven days onward. On the other hand, in S plants there were no significant differences between control and Ni-treated plants (independently of the concentration) for ΦPSII , ETR and qP . Furthermore, in S plants the presence of Ni in the substrate led to an increase in qN , which raised by around 50% compared to controls after seven days, remaining relatively constant until the end of the experiment.

Stomatal density and pore size were measured in both the accessions at the beginning of the treatment. Plants showed the same stomatal density for the leaf lower and upper side (around 20 ± 2 and 22 ± 3 stomata per 0.361 mm^2 leaf area, in NS and S accession, respectively) without any significant difference between the accession, as well as in the case of pore size (around $35 \pm 1 \mu\text{m}$ for both the leaf lower and the upper sides).

Table 4 (a) Growth rate of the two *S. paradoxa* accessions, calculated as the angular coefficient (slope, cm day⁻¹) of the linear regression between plant length increment and sampling time in plants treated with increasing NiSO₄ for 27 days. (b) Shoot:root ratio in Ni concentration of two *S. paradoxa* accessions, calculated as the angular coefficient of the linear regression between Ni shoot concentration and Ni root concentration. Values are means of 12 replicates $\pm$ standard deviations. Letters indicate significant differences among all the samples according to the Tukey's test ($p < 0.05$).

Accession	R	p value	Slope	
a) Growth rate				
Non-serpentine				
Cont	0.9683	0.0001	0.3590	$\pm$ 0.01560 f
40	0.9471	0.0001	0.2160	$\pm$ 0.0108 c
20	0.9222	0.000	0.1518	$\pm$ 0.0094 b
60	0.8546	0.0001	0.02505	$\pm$ 0.0022 a
Serpentine				
Cont	0.9671	0.0001	0.2550	$\pm$ 0.0098 d
20	0.9622	0.0001	0.4400	$\pm$ 0.0183 g
40	0.9729	0.0001	0.3262	$\pm$ 0.0114 e
60	0.9745	0.0001	0.2653	$\pm$ 0.0089 d
b) Shoot:root ratio in Ni concentration				
Non-serpentine				
20	0.9801	< 0.0001	0.0743	$\pm$ 0.0022 a
40	0.9820	< 0.0001	0.0903	$\pm$ 0.0025 ab
60	0.9518	< 0.0001	0.0958	$\pm$ 0.0045 b
Serpentine				
20	0.9218	< 0.0001	0.0747	$\pm$ 0.0046 a
40	0.9379	< 0.0001	0.1121	$\pm$ 0.0061 c
60	0.9850	< 0.0001	0.1020	$\pm$ 0.0026 bc

Discussion

Serpentine accessions showed enhanced Ni tolerance

The dose-dependent negative effect of Ni excess on root growth showed significant variation among accessions, with declines in root elongation in NS plants starting at lower NiSO₄ concentrations compared to S plants. Interestingly, at low Ni not only the S accessions were not affected, but they generally showed improved growth, although differences were not significant. To date, a stimulatory effect of Ni concentrations exceeding the threshold considered toxic for non-metallophytes (5 μ M, Marschner 1995) has only been documented for hyperaccumulators. This phenomenon has been partly attributed to

hyperaccumulation-mediated cytosolic Ni depletion in the low-dose range (Bettarini et al. 2021; Colzi et al. 2023), generating high metal requirement for growth (Cappa and Pilon-Smits 2014, Manara et al., 2020). In the present study, a similar stimulatory effect was observed, although with a much weaker intensity and at significantly lower Ni concentrations compared to hyperaccumulators. Thus, our data suggest that even in excluding serpentinophytes metals that are micronutrients, such as Ni (Dixon et al 1975), may have a stimulatory effect. At low Ni concentrations in the substrate, root level exclusion mechanisms could result in a metal deficiency at the whole plant level, generating a Ni requirement for optimal growth that is higher than sensitive plants but lower than in hyperaccumulators (Bettarini et al. 2021; Colzi et al. 2023).

All the dose–response curves exhibited a significant fit to the logistic model, and tolerance parameters varied significantly among accessions and between the two edaphic groups. The EC50_{ext} values of all S plants (mean around 60 μ M) were significantly higher than those of NS plants (mean around 30 μ M), demonstrating their enhanced Ni-tolerance. Our findings on tolerance in excluders, and the subsequent ones regarding Ni exclusion, represent the first solid insights derived from a comparison of a substantial number of contrasting accessions, given the limits of the above-mentioned literature. Our dataset on *Silene paradoxa* indicates that the colonisation of serpentine substrates requires the evolution of enhanced Ni tolerance, supporting the idea of local adaptation within individual populations rather than adaptive diversity, as already hypothesised by some limited studies on other serpentinophytes (Kolář et al. 2014a; Veatch-Blohm et al. 2017; Burrell et al. 2012; Pakdaman et al. 2013; Koszelnik-Leszek and Bielecki 2013). Therefore, it is likely that this adaptation influenced the ecological distribution of this species on such harsh substrates, likely as a result of the selective pressure imposed by prolonged exposure to high soil Ni, a factor considered to be one of the drivers of serpentine adaptation (Brooks 1987; Brady et al. 2005; Kazakou et al. 2008). Despite this, there are presumed exceptions of constitutive Ni tolerance in *S. dioica* (Westerbergh 1994) and in the “pre-adapted” species *Galium valdepiusolum* (Kolář et al. 2014b). Interestingly, in *S. paradoxa*, the accessions from the outcrop with the lowest Ni concentration also

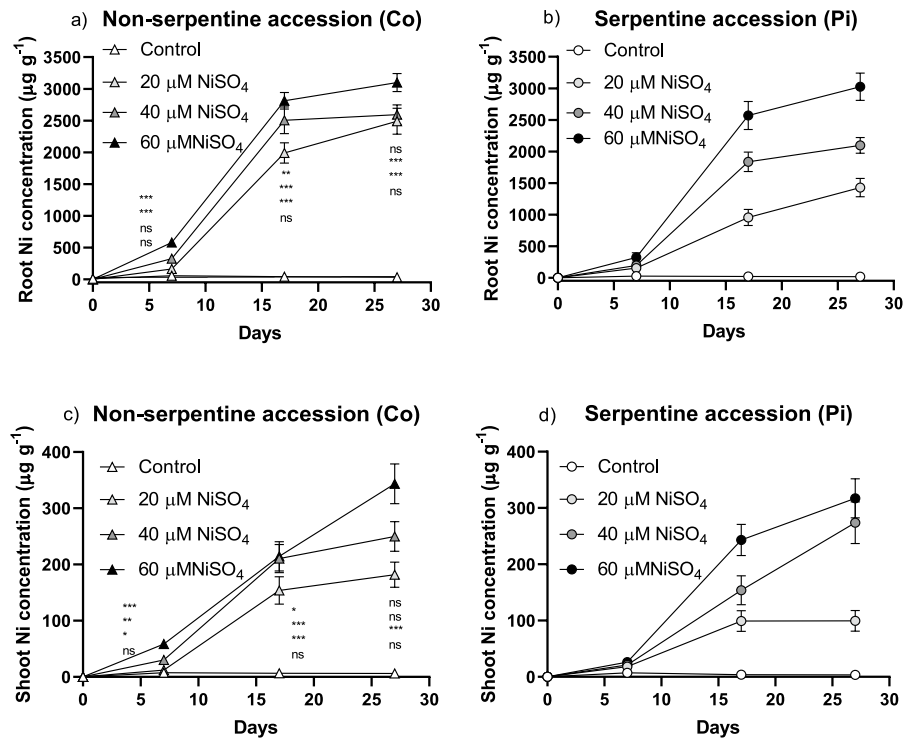


Fig. 4 Root and shoot Ni accumulation of two accessions of *S. paradoxa* treated with increasing NiSO₄ concentrations for 27 days. a) Ni concentration in roots of the non-serpentine accession, b) Ni concentration in roots of the serpentine accession, c) Ni concentration in shoots of the non-serpentine accession, d) Ni concentration in shoots of the serpentine accession. Values are means of 12 replicates $\pm$ standard deviation. Aster-

isks indicate significant differences according to Tukey's test of each treatment concentration at each data point between the two accessions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (the sequence of the indications reflects the one of the treatments: 60 µM NiSO₄, 40 µM NiSO₄, 20 µM NiSO₄ and control condition)

exhibited the lowest EC50_{ext} value. This suggest a potential positive relationship between tolerance and Ni concentration in the substrate of origin, as reported for some Ni hyperaccumulators (Seregin et al. 2022), although this is not always the case (e.g. see Galardi et al. 2007; Bettarini et al. 2021).

Serpentine accessions showed enhanced Ni exclusion but not enhanced internal Ni tolerance

Nickel accumulation increased in a dose-dependent manner, with root concentrations always higher than those in shoots for all accessions. This generated a translocation coefficient under the unit, typical of excluders (Wójcik et al. 2017). To compare the levels of accumulated metal in the roots of all accessions, we calculated the tolerance parameter based on internal Ni concentration (EC50_{int}) as it is considered as

a proxy of the accumulation capacity (Galardi et al. 2007; Bettarini et al. 2021; Colzi et al. 2023). Unlike EC50_{ext}, EC50_{int} varied within S and NS accessions, although with similar mean values, thus suggesting no significant effect of the substrates of origin. Therefore, in S plants, Ni tolerance depended on the ability to limit the entry of the metal in the root symplast rather than on an increased tissue tolerance. Furthermore, the similar translocation coefficients between S and NS plants suggest that also exclusion from the shoots did not play a major role in tolerance mechanisms of *S. paradoxa*. However, despite the almost identical translocation coefficient among S plants, some variation in exclusion behaviour and detoxification mechanisms cannot be excluded due to observed differences in shoot Ni accumulation.

The reliability of our estimation of the translocation coefficient was ensured by two key factors:

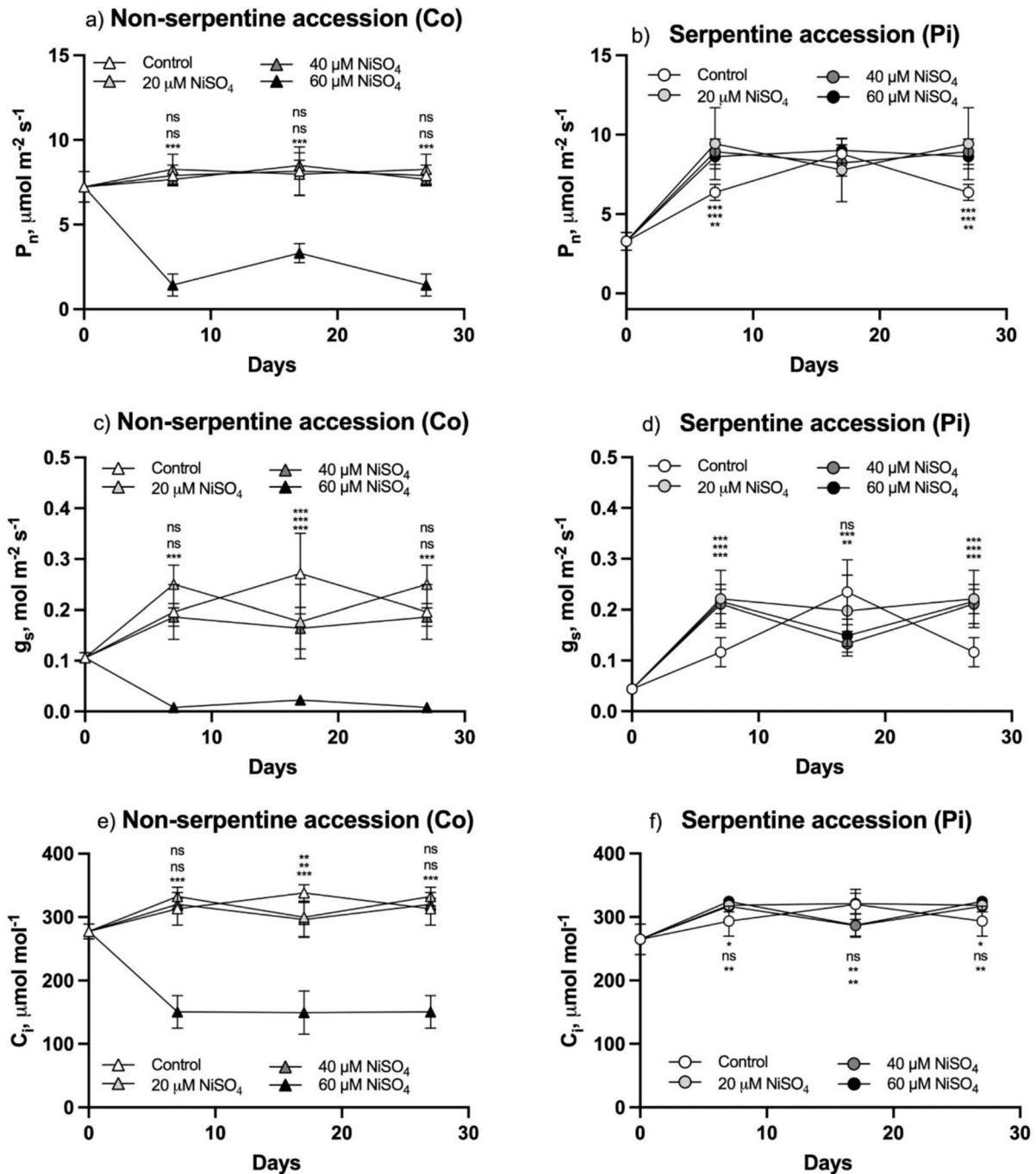


Fig. 5 Time dependent changes in gas exchange parameters in two *S. paradoxa* accessions treated with increasing $NiSO_4$ concentrations for 27 days. a) P_n in the non-serpentine accession, b) P_n in the serpentine accession, c) g_s in the non-serpentine accession, d) g_s in the serpentine accession, e) C_i in the non-serpentine accession, f) C_i in the serpentine accession. Values

are means of 6 replicates $\pm$ standard deviation. Asterisks indicate significant differences according to Dunnett's test of each treatment concentration at each data point in each accession. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (the sequence of the indications reflects differences compared to control in the 20, 40 and 60 μM $NiSO_4$ treatment respectively)

(i) its calculation through linear regression, which confirmed that plants were not reacting to metal toxicity by decreasing its translocation; and (ii) the analysis of the Ni symplastic fraction of the roots (done via the appropriate desorption of the organ) prevented bias due to a differential amount of metal bound to the cell wall, which can potentially vary across accessions but also lead to underestimate the coefficient itself.

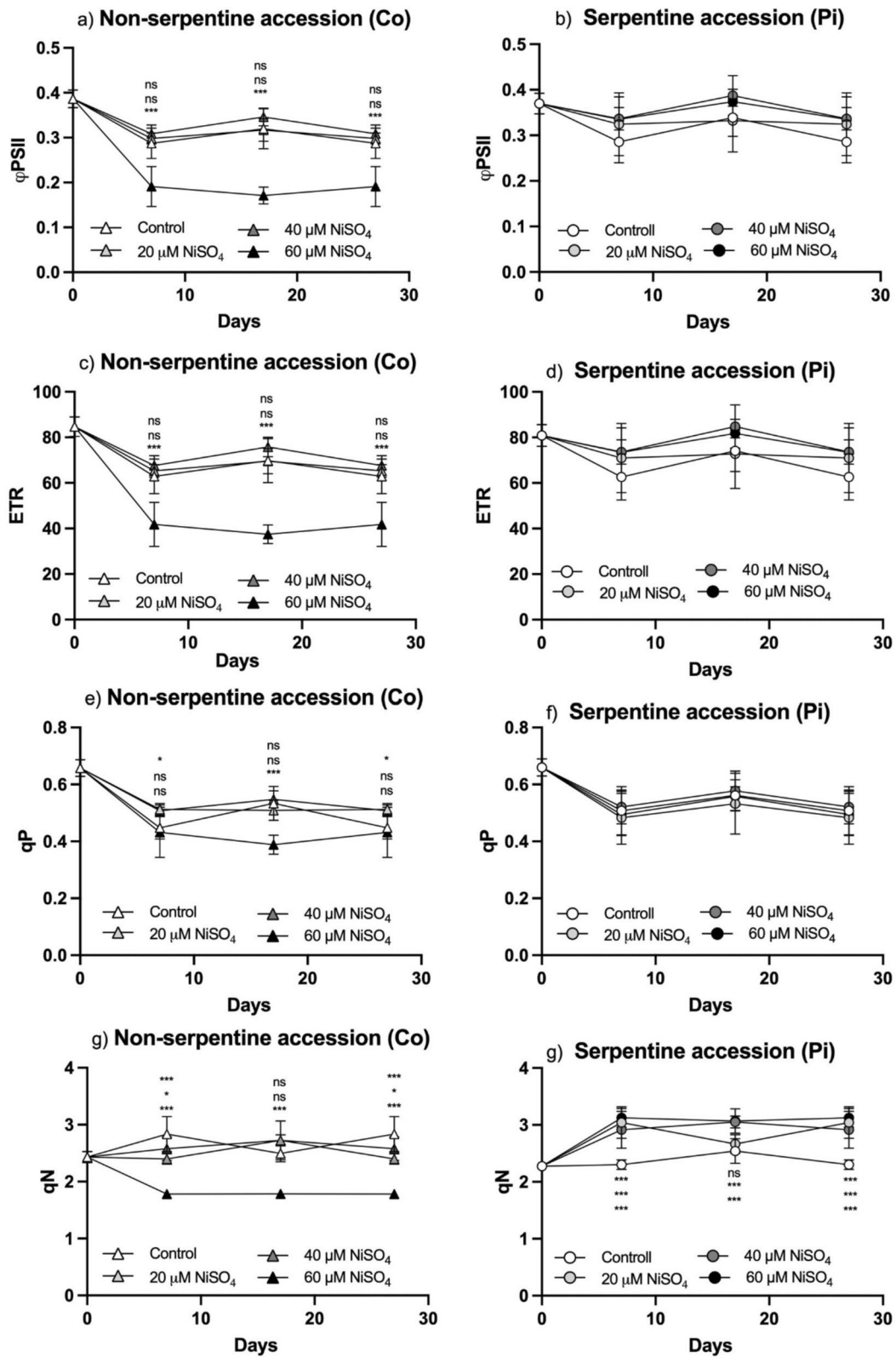
Our data revealed that different Ni tolerance were linked with varying degree of root Ni exclusion in the two contrasting plant groups. Without data modelling to calculate and compare tolerance parameters and translocation coefficients in S and NS plants, it would have been impossible to unveil the real features of metal adaptation. A simple comparison of growth and accumulation data would have merely highlighted lower Ni concentrations in tolerant accessions, overlooking crucial nuances. Furthermore, our data demonstrated that variation in both Ni-induced growth responses and accumulation patterns can naturally occur within NS and S accessions, thus highlighting the risk of misleading results when making a blind choice in selecting a single pair of accessions for comparison.

In other serpentine metal excluders, highly Ni tolerant accessions have been found to accumulate: (i) less Ni compared to non-serpentine ones (Kolář et al. 2014a; Veatch-Blohm et al. 2017); (ii) similar levels (Burrell et al. 2012; Pakdaman et al. 2013); or (iii) even higher ones (Koszelnik-Leszek and Bielecki 2013). This suggests the internal tolerance may play a key role on some occasion, as for example when measured Ni concentrations were higher than those in non-serpentine accessions. Nevertheless, our data on *S. paradoxa* suggested that adaptation to serpentine habitats relies primarily on exclusion mechanisms and, potentially, the advantage of a lower cost of tolerance compared to internal detoxification mechanisms. In any case, environmental factors are also likely to contribute to metal exclusion and, therefore, adaptation. For example, interactions between elements present in serpentine soils could influence Ni tolerance, as reported for the Ca-mediated reduction of Ni toxicity and accumulation in the serpentine excluder *S. italica* (Gabbriellini et al. 1990) and for *K. arvensis* with Mg (Kolář et al. 2014a).

Ni induced changes in gas exchanges in non-serpentine plants

To delve deeper into the physiological mechanisms underlying the differential response to external Ni in S and NS plants, we analysed Ni effect on photosynthetic performance. This was done by selecting two contrasting accessions providing an *ad-hoc* comparative system in which the S plants seemed to rely mainly on Ni exclusion and not on Ni internal detoxification mechanisms. Confirming the results obtained in experiment 1, the greater Ni sensitivity of NS plants led to a negative effect on root elongation in a dose-dependent effect, whereas in the S accession elongation rates improved at the two lowest concentrations and declined only with 60 μM NiSO_4 . This confirmed the stimulatory effect of low Ni doses, which, while evident but statistically not significant in the short-term (7-day) experiment, became pronounced and statistically different after a longer exposure time (more than two weeks), thus disclosing an interesting feature of excluding plants. As for Ni accumulation, the concentrations accumulated in S plants were lower, in most of cases significantly, than those in NS plants, but calculations of shoot:root ratio confirmed that reduced translocation was not involved in the exclusion mechanisms in metal tolerant plants. In any case, the fundamentally important outcome was that, for most of the concentrations used and times applied, the plants could be considered still growing and, therefore, metabolically efficient to produce Ni-imposed responses.

Leaf gas exchange and chlorophyll a parameters highlighted contrasting Ni-induced patterns in NS and S plants, with interesting differences between the two accessions emerging with the highest Ni concentration tested. Indeed, in S plants, the presence of Ni, even 60 μM NiSO_4 , generally improved P_n and g_s , resulting in small but significant increases in C_i . Chlorophyll fluorescence in S plants also indicated that, while NiSO_4 treatment did not alter most of the measured parameters, it led to a significant increase in qN , indicating a greater energy allocation towards the non-photochemical pathway and possibly an increased photoprotection through the xanthophyll-II cycle (Adams et al. 1995; Liu et al. 2014). Overall, both C_i and g_s data suggest that under control conditions, stomatal opening limited photosynthesis of S plants and that part of beneficial effects



◀Fig. 6 Time dependent changes in chlorophyll a fluorescence parameters in light adapted leaves of two *S. paradoxa* accessions treated with increasing NiSO_4 concentrations for 27 days. a) ΦPSII in the non-serpentine accession, b) ΦPSII in the serpentine accession, c) ETR in the non-serpentine accession, d) ETR in the serpentine accession, e) qP in the non-serpentine accession, f) qP in the serpentine accession, g) qN in the non-serpentine accession h) qN in the serpentine accession. Values are means of 6 replicates $\pm$ standard deviation. Asterisks indicate significant differences according to Dunnett's test of each treatment concentration at each data point in each accession. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (the sequence of the indications reflects differences compared to control in the 20, 40 and 60 μM NiSO_4 treatment respectively)

observed in Ni-treated S samples could be attributed to an enhanced stomatal opening. By contrast, in NS plants, 60 μM NiSO_4 quickly (seven days) decreased P_n , g_s and C_i and most of the measured chlorophyll fluorescence parameters, with the effects lasting for the whole duration of the experiment. These results indicated that the observed declines in photosynthesis were in part driven by stomatal closure, as conductance rates dropped by more than 95% compared to control plants, thus severely affecting CO_2 capture and reducing both C_i and P_n . However, the declines in gas exchange parameters in NS plants were also associated with lower actual quantum yield of PSII (ΦPSII) and ETR compared to controls, evident already seven days after Ni addition and remaining consistently lower throughout the experiment. These observed declines in P_n were more pronounced than those in ETR (82% vs 34% declines compared to controls), indicating an increased ratio of PSII activity per CO_2 fixed and an increase in photorespiration due to a decrease in CO_2 supply to Rubisco (Lal et al. 1996). Furthermore, ΦPSII and ETR declines could also indicate adjustments to cope with the limited consumption of ATP and NADPH as a result of the low C_i (Bussotti et al. 2012). Our hypothesis was supported by the fact that ΦPSII and ETR declines did not significantly alter photochemical quenching (qP), but significantly reduced energy dissipation via non-photochemical pathways (qN) compared to control plants. Interestingly our data indicated that, given the far greater declines in g_s compared to P_n and the decline in C_i , the presence of Ni excess in NS plants led to strong stomatal limitations. As previously shown (Rucińska-Sobkowiak 2016), it is therefore possible to speculate that in Ni-sensitive NS plants the presence of excessive Ni affected water status in

metal-treated plants by altering water absorption (e.g. reduction in root surface area, changes in root anatomy and/or morphology) or affecting the efficiency of the water flow from the roots to the shoots. The opposite trend occurred in the S plants where the presence of Ni improved stomatal opening compared to controls, probably as a mere result of the preserved, or even ameliorated, root growth (and consequently functioning) in presence of the metal, as reported above and ascribed to the Ni exclusion mechanisms per se.

Conclusion

Our study on contrasting accessions of the facultative serpentinophyte *Silene paradoxa* suggests that adaptation to serpentine substrates can require the evolution of enhanced Ni tolerance due to the selective pressure imposed by high soil Ni concentrations. Furthermore, accumulation data indicate that Ni tolerance depends on the ability to limit metal entry in the root symplast rather than being associated with enhanced tissue tolerance or shoot exclusion. Finally, Ni excess, rather than affecting leaf photochemistry per se, differentially affected stomatal opening. This suggests that the preservation of the photosynthetic activity in serpentine accession relied more on maintaining adequate stomatal opening in the presence of the metal rather than on an increased Ni tolerance in photosynthetic tissues. Thus, metal exclusion was confirmed to be the main Ni tolerance mechanism in facultative metallophytes such as *S. paradoxa*.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

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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