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REGULAR ARTICLE

Root foraging and avoidance in hyperaccumulator and excluder plants: a rhizotron experiment

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Abstract
Aims Metal hyperaccumulation is a rare phenomenon described for an increasing number of plant taxa. In this study we investigated the root growth responses of the well-known nickel, zinc, cadmium hyperaccumulator *Noccaea caerulescens* and of the metal tolerant (non-accumulator) *Stellaria media*, in order to observe root foraging vs avoidance responses to nickel.
Methods To allow for observations of root growth and foraging preferences, two accessions of *Noccaea caerulescens* and two accessions of *Stellaria media* orginating from high nickel and low nickel habitats were grown in rhizotrons with localized nickel enrichment.
Results The root density in the control and nickel-enriched soil areas in the rhizotrons with different *N. caerulescens* accessions had distinct responses: moderate nickel avoidance was recorded for the non-nickel accession, while a clear foraging response was observed in *N. caerulescens* from the nickel accession. In contrast, nickel rooting avoidance was observed for both *S. media* accessions and was more pronounced in the non-nickel accession.
Conclusions This study shows that *N. caerulescens* originating from different accessions responded differently to soil nickel enrichment, with the nickel accession of *N. caerulescens* actively foraging for nickel, suggesting a physiological adaptation and demand for this metal. In contrast, a clear nickel avoidance response by a metal tolerant species*, S. media*, was observed in this study, a phenomenon which has not been previously

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described; this suggests that root avoidance responses might play a role in the adaptation of metal tolerant species to Ni-rich soils.

Keywords Nickel · Hyperaccumulation · Metal tolerance · Root foraging · Root avoidance

Introduction

Plants colonizing metalliferous soils ('metallophytes') have evolved physiological mechanisms which enable them to tolerate metal toxicity at the plant level (Baker 1981). These mechanisms to cope with extremely large (toxic) metal concentrations in their growth medium can be classed in three categories: (i) excluders (ii) indicators and (iii) (hyper) accumulators (Baker 1981). In excluder plants, metal concentrations in the shoot are maintained constant and low over a wide range of soil metal concentration gradients (Baker 1981). Conversely, metal hyperaccumulators are able to accumulate extraordinarily high concentrations of specific metals or metalloids into their shoots (Baker and Brooks 1989; Reeves and Baker 2000; van der Ent et al. 2013). Hyperaccumulator plants can achieve such extreme levels of foliar sequestration due to enhanced uptake and translocation mechanisms (Baker 1981, 1987); with threshold levels of 1000 $\mu g\ g^{-1}$ for nickel (Ni), 300 $\mu g\ g^{-1}$ for cobalt (Co), 3000 $\mu g\ g^{-1}$ for zinc (Zn) and 10,000 $\mu g\ g^{-1}$ dry weight for manganese (Mn) (Baker and Brooks 1989; van der Ent et al. 2013). Metal hyperaccumulator plants have received more attention than metal excluders due to their potential for applications in the remediation of metal-contaminated soils (Baker et al. 1994; Lombi et al. 2000; Krämer 2005; Wenzel et al. 1999). Among the over 700 metal hyperaccumulator plants currently known (Baker and Brooks 1989; van der Ent et al. 2013), most research to date has focused on small biennial or short-lived perennial European species that have a large natural variation for trace metal hyperaccumulation, specifically: *Noccaea* (synonym *Thlaspi*) *caerulescens* (J.Presl & C.Presl) F.K.Mey. (Gonneau et al. 2014; Schwartz et al. 2006) and *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz. (Verbruggen et al. 2009, 2013; Krämer 2010; Hanikenne and Nouet 2011; Meyer et al. 2015). Despite being an uncommon species, *N. caerulescens* can be found over a widespread area of central Europe, ranging from Poland and the Czech Republic in the east, west to Great Britain and south to northern Spain, and to the Alps on the border of France and Italy (Reeves et al. 2001). A peculiarity of this species is its occurrence on a variety of different substrates, ranging from uncontaminated "normal" soils to soils naturally enriched or contaminated soils with high concentrations of certain trace metals such as Ni, Zn and Cd. Even if occurring on uncontaminated soils, many populations of *N. caerulescens* can tolerate conditions on sites with anthropogenic metal pollution (Baker et al. 1994; Ingrouille and Smirnoff 1986; Gonneau et al. 2017; Meerts and Isacker 1997), including mine waste and smelter sites (Reeves et al. 2001); while other populations occur on Ni-rich soils developed from ultramafic bedrocks in Europe (Meyer 2006; Reeves et al. 2001; Sterckeman et al. 2017).

Following its adaptation spectrum, *N. caerulescens* is a Ni hyperaccumulator in ultramafic accessions, but can also be a Zn (Baker and Brooks 1989) and Cd (Brown et al. 1995; Hutchinson et al. 2000) hyperaccumulator when growing on Zn-Cd-Pb natural ('calamine') mineralised outcrops or contaminated sites (Assunção et al. 2003a; Callahan et al. 2016; Escarré et al. 2013; Gonneau et al. 2014; Peer et al. 2003). This ability to tolerate and accumulate several different metals likely reflects the relatively low specificity of some mechanisms involved in metal transport and chelation in this species (Merlot et al. 2018) and suggests that highly efficient mechanisms of foraging and uptake of these metals may be involved (Haines 2002). Despite the contribution of recent studies to elucidate plant-internal processes associated with metal hyperaccumulation (Assunção et al. 2003b; Brooks 1998; Krämer et al. 1996, 1997, 2000; Krämer 2010; Lombi et al. 2000), increased knowledge about rhizosphere processes (Dessureault-Rompré et al. 2010; Wenzel et al. 1999, 2003), the precise mechanisms by which hyperaccumulator plants take up metals from the soil is still relatively limited. Being a hyperaccumulator of different metals (Ni, Zn, Cd), *N. caerulescens* is receiving considerable scientific attention with the recognition of its usefulness in ecological, physiological and genetics and molecular biological studies of metal accumulation (Baker et al. 1994; Craciun et al. 2012; Halimaa et al. 2014; Lasat et al. 1996; Milner and Kochian 2008; Milner et al. 2012; Puschenreiter et al.

2003; Vàzquez et al. 1992), and the key role of metal-accumulating plants in remediating metal-contaminated soils using phytotechnologies (Baker et al. 1991; Brown et al. 1994; Escarré et al. 2000; Hammer and Keller 2003; Jacobs et al. 2017, 2018; McGrath et al. 1993, 2006; Robinson et al. 1998; Schwartz et al. 2003).

Understanding root responses is essential to improve knowledge of the physiological processes responsable for hyperaccumulation. The capacity of roots for differential growth towards various micro-environments has been shown for a range of different plant species (Haines 2002), but is one of the least well understood facets of plant life (Robinson 1994). Plants tend to concentrate roots (e.g. forage) in soil patches in which resources are more abundant (Fitter 1994), and proliferate lateral roots preferentially in nutrient-rich zones to access nutrients in diverse soil microenvironments (Guan et al. 2014). Thus, the major function of root foraging responses in higher plants is to explore and access essential resources, such as nutrients and water, that contribute to plant growth and development (Casper and Jackson 1997; Hodge 2004; Hutchings and de Kroon 1994; Liu et al. 2010; Robinson 1994; Schenk et al. 1999). Interestingly, root foraging responses were also reported for hyperaccumulator plants in the presence of certain trace metals such as Zn, Cd and Ni (Haines 2002; Liu et al. 2010; Schwartz et al. 1999; Whiting et al. 2000; Dechamps et al. 2008), suggesting that hyperaccumulator plants might have a specific requirement for these metals. Localized root proliferation (root foraging) is also listed as one of the most likely adaptations explaining the highly efficient metal uptake in *N. caerulescens* (Haines 2002; Schwartz et al. 1999; Whiting et al. 2000). Most experiments of root responses in relation to spatially localized resources in soil have so far concentrated on nutrients such a NO_3^-, NH_4^+, P and K (Hodge 2004; Guan et al. 2014; Robinson 1994), with only a few studies focussing on trace metals such as Zn (Haines 2002; Schwartz et al. 1999; Whiting et al. 2000; Dechamps et al. 2008; Saison et al. 2004), Cd (Liu et al. 2010; Schwartz et al. 2003; Whiting et al. 2000) and Ni (Dechamps et al. 2008). Previous studies on root foraging conducted with *N. caerulescens* have investigated the Zn foraging responses of this species, and positive root proliferation has been observed in response to substrate patches with high Zn concentrations (Dechamps et al. 2008; Schwartz et al. 1999; Whiting et al. 2000). Root responses of hyperaccumulator plant species in the presence of localized Ni enrichments were investigated in detail by Dechamps et al. (2008), where preferential root allocation on Ni-enriched soil compartments was observed in a calamine *N. caerulescens* accession. Furthermore, Moradi et al. (2009) studied Ni foraging responses in the Ni hyperaccumulator species *Berkheya coddii*, without observing any preferential root allocation in response to soil Ni. To the best of our knowledge, root responses of excluder plants to Ni have not been previously investigated.

The aims of this study were to address the following questions: i) Does the Ni hyperaccumulator *N. caerulescens* preferentially forage in Ni-enriched zones? ii) Does a positive root response to Ni enhance metal accumulation in *N. caerulescens*? iii) Do excluder species have different root responses to Ni soil enrichment compared to hyperaccumulators? iv) Do different accessions of the same plant species respond differently to the presence of Ni in the soil? In order to address these questions, we have investigated the root responses of *N. caerulescens* in establish active Ni foraging vs avoidance strategies. The metal tolerant *Stellaria media* (L.) Vill. (Caryophyllaceae) was included in this experiment, to compare the root responses of *N. caerulescens* to a Ni excluder (non-accumulator) species. In order to allow for observations of root growth and foraging preferences, the tested plants were grown in rhizotrons with localized Ni enrichments. Nickel and non-Ni accession of each plant species were investigated to conduct comparative studies in terms of their relative root foraging responses.

Methods

Plant species

The Ni, Zn and Cd hyperaccumulator *N. caerulescens* and the metal tolerant (but excluder) species *Stellaria media* were selected for the experiment. Two accessions of each species, adapted to either high Ni or low Ni soils were used in the experiments . The high Ni accession of *N. caerulescens* was obtained from an ultramafic outcrop near Bergenbach, France (47°54′26”N 6°57′35″E) (Chardot et al. 2007; Escarré et al. 2013; Reeves et al. 2001). Reported total near surface soil Ni concentrations in this site are in the range of 469 to 1301 mg kg^{-1} (Reeves et al. 2001) and up to 2500 mg kg^{-1} in the lower soil horizons (Chardot et al. 2007). The low Ni accession of *N. caerulescens* was obtained from the old Zn-Pb mine of Plombieres, Belgium (50°44′04”N 5°57′53″ E), a calamine site which has long been known for its remarkable metal-tolerant flora (Meerts and Isacker 1997; Simon 1975) and where *N. caerulescens* is a Zn hyperaccumulator. Accessions of *S. media* were obtained from the Ni-rich ultramafic area of Monte Prinzera (Italy, 44°38'25.2"N 10°05'32.5"E), a site with a Ni total concentration of 2466 mg kg^{-1} (Lombini et al. 1998), as well as from the non-metalliferous site of Ticino (Italy, 45°17'57.2"N 8°56'55.9"E). Accessions of each species were obtained from seed material collected in the field from different plant individuals.

Experimental setup

Soil preparation and analyses

A garden soil characterized by low total and plant available Ni concentrations was selected for the experiment (Table 1). The selected soil had relatively high Zn concentrations (Table 1) which is particularly advantageous as *N. caerulescens* has a high constitutive requirement for Zn and a greater sensitivity to Zn deficiency than non-accumulator plants (McGrath et al. 1997; Ozturk et al. 2003; Shen et al. 1997); and this experiment aimed to reveal root responses to Ni while Zn supply is not limiting. A further selection criterion for the soil was its dark colour, in order to facilitate imaging analyses of root distribution based on colour contrast of roots/background (see “Root density analyses” section). The soil was oven dried at 40 °C for 24 h, sieved at <1 mm and divided in equal parts of 1.5 kg. Two aliquots of soil were enriched with Ni, each with a specific Ni form and the remaining soil was kept as a control. Two Ni forms with low solubility were chosen for the soil enrichment, in order to avoid substantial Ni diffusion in the control soil: i) a pure natural garnierite mineral ($(Ni,Mg)_3(Si_2O_5)(OH)_4$; 20% Ni) obtained from New Caledonia, ground and sieved to <0.5 mm and ii) Ni carbonate chemical ($Ni_4CO_3(OH)_6(H_2O)_4$, 99.9%, Sigma-Aldrich). An amount of each Ni compound was added to the soil in order to obtain a nominal Ni concentration of 2000 mg kg^{-1} to resemble typical total Ni concentrations of natural ultramafic soils. Moist soil was autoclaved, mixed with the Ni forms and manually homogenized, then oven dried at 40 °C, re-moistened and re-dried along 10 days to allow equilibration. Half of the untreated soil to be used as control was limed with 0.2% *w*/w $CaCO_3$ in order to obtain the same pH as in the soil spiked with Ni carbonate, to avoid interference of other factors such as differences in nutrient availability among control and Ni carbonate spiked soil driven by different pH values. Soil pH was measured with a pH-meter after mixing soil with ultra-pure water with a 1:2.5 ratio *w*/*v* and performed in two replicates. Texture was measured following the wet sieving sedimentation method. Soil TOC and TN content were measured via combustion analyses (Dumas method) with a Vario Macro Cube instrument (Elementar). Available phosphorous was determined according to the Olsen-P method (Olsen et al. 1954) followed by molybdate blue colorimetry (Murphy and Riley 1962). A subsample of the control soil as well as the limed and Ni spiked soils were digested in HCl 37% and HNO_3 65% at 1:3 ratio (*aqua regia*) in a digestion block at 155 °C for 3 h to determine pseudo-total element concentrations. Plant available metal fractions were determined through DTPA (diethylene triamine pentaacetic acid) (Lindsay and Norvell 1978) extractions. Digested samples were measured for macronutrients with ICP-OES (Optima 8300, Perkin Elmer) and for trace metals with ICP-MS (Elan 9000 DRCe, Perkin Elmer). DTPA extracts were measured with ICP-MS (Elan 9000 DRCe, Perkin Elmer).

Table 1 Pseudo-total, DTPA-extractable element concentrations, pH, Olsen-P, TOC, TN and texture of the control and Ni-spiked (garnierite and Ni-carbonate) soils

		Control soil	Garnierite	Ni-carbonate
Ca_{total}	$g\ kg^{-1}$	6.91 ± 2.5	–	–
Mg_{totoal}	$g\ kg^{-1}$	1.76 ± 1.6	–	–
K_{total}	$g\ kg^{-1}$	3.04 ± 1.1	–	–
Fe_{total}	$g\ kg^{-1}$	24.3 ± 1.6	–	–
Mn_{total}	$mg\ kg^{-1}$	194 ± 2.7	–	–
Zn_{total}	$mg\ kg^{-1}$	422 ± 0.8	–	–
Zn_{DTPA}	$mg\ kg^{-1}$	100 ± 3.1	–	–
Ni_{Total}	$mg\ kg^{-1}$	9.36 ± 21	1987 ± 15	2006 ± 3.1
Ni_{DTPA}	$mg\ kg^{-1}$	0.454 ± 8.9	3.63 ± 0.6	984 ± 1.0
Olsen-P	$mg\ kg^{-1}$	49.3 ± 3.3	–	–
pH		6.98 ± 0.1	7.01 ± 0.1	7.43 ± 0.1
TOC	**wt%**	8.71 ± 1.8	–	–
TN	**wt%**	0.50 ± 3.4	–	–
Texture		Sandy loam		

Data show average values ± relative standard deviation % ($n = 3$)

Rhizotron setup

In order to observe root growth responses of the tested plant species in the presence or absence of Ni, a rhizotron experiment was conducted. Rhizotrons consist of boxes with a transparent window filled with soil, which allow for non-destructive observations of roots on a transparent surface (Smit et al. 2000). Among the various types of methods described for investigating localized supply of nutrients or other elements, Robinson (1994) defined as ideal a system where no physical barriers are imposed to root growth. Therefore, we have chosen to setup a rhizotron experiment growing the tested plants on a substrate without any physical barrier between the control soil and the Ni enriched soil. Self-made rhizotrons (Fig. 1) were constructed from Petri dishes (12 × 12 cm), which were filled in with paraffine in order to reduce their thickness to 0.5 cm. Openings for the seedlings transplantation and for watering were created on the upper part of the Petri dishes. In order to fill in the rhizotrons with a control soil (left side) and the Ni enriched soil (right side as in Whiting et al. 2000), a plastic foil was used to create a vertical separation, which was subsequently removed. Limed soil was used as a control for the Ni carbonate treatments. The soil surface on the whole rhizotrons was then carefully compacted to avoid inhomogeneities to appear during the plant growth, as well as to allow for precise root observations. Seedlings were prepared on a vermiculite and quartz sand mix and transplanted into the rhizotrons as soon as cotyledons had appeared. Rhizotrons were then closed with the Petri dish cover plates, wrapped on the outside with aluminium foil to protect the roots from light and set up at an inclination of 45° with the rooted surface facing down. The two Ni forms (carbonate and garnierite mineral) were tested for each plant accession of *N. caerulescens* and *S. media* and for each treatment three replicates were included for a total of 24 rhizotrons.

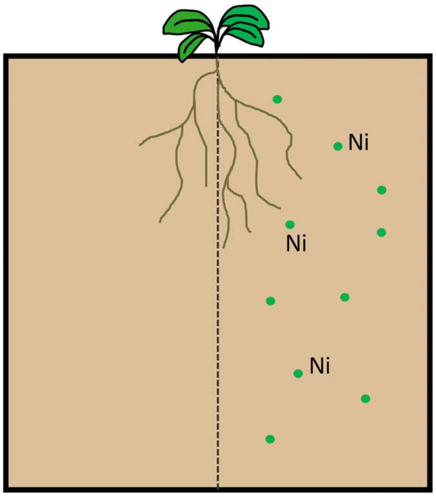

Fig. 1 Schematic view of a rhizotron filled in with a control soil on the left side and with Ni enriched soil on the right side

The experiment was conducted for 3 weeks in a growth cabinet with a 12-h/day of light, a temperature of 20–25 °C (day-night), 75% humidity and light intensity of 350 μmol m^2 sec^{-1} photosynthetically active radiation (PAR).

Plant biomass analyses

Plant shoots were harvested after 3 weeks, carefully rinsed with deionized water and dried at 65 °C for 24 h. The shoots were then weighed, grinded and digested in a mixture of HNO_3 and H_2O_2 (with a ratio 5:1) in a digestion block at 115 °C for 158 min (Zhao et al. 1994). Pseudo-total element concentrations in the plant digests were measured with ICP-MS (Elan 9000 DRCe, Perkin Elmer) and with ICP-OES (Optima 8300, Perkin Elmer).

Root density analyses

Pixel count method

At the end of the growing period (3 weeks) and before the harvesting of shoots, high resolution images of all rhizotrons were taken with a Canon 5D MkII (24-megapixel full-frame) camera with 50 mm prime lens. The images were then processed with the imaging software Image-J and converted to binary, where only black ("0") and white ("255") pixels were displayed (Fig. 2). The colours of the pictures were then inverted in order to obtain black roots on a white background. In the binary images, "0" (black/roots) and "255" (white/bulk soil) pixels were counted in each half of the rhizotrons with the Image-J program function "pixel count". Root density in the two areas of the rhizotrons was than measured as the percentage (%) of black pixels (roots) in each half calculated from the total black pixels on the full surface.

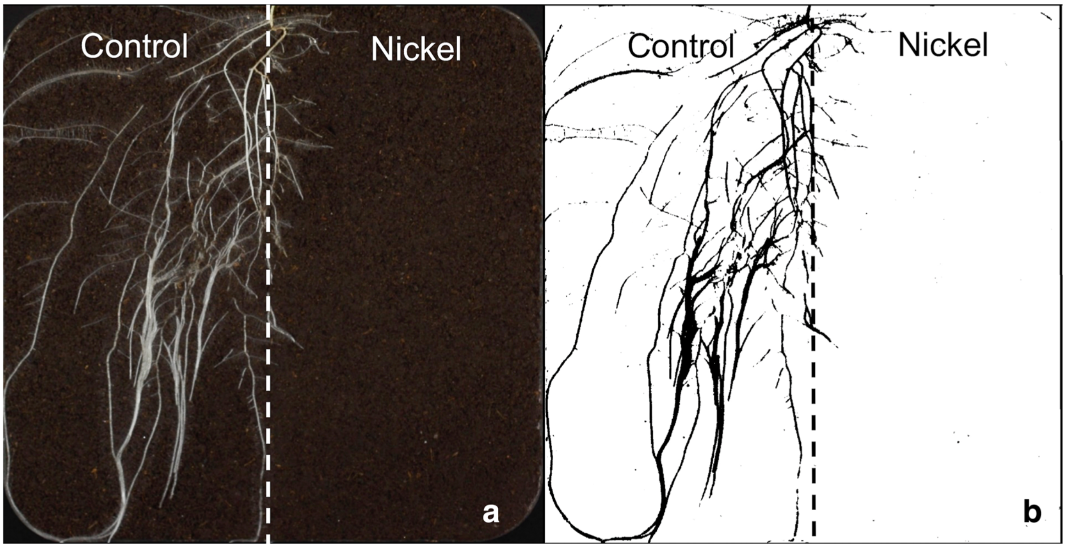

Fig. 2 Rhizotron rooted surface at the end of the experiment: **a** original picture and **b** converted to “bits” (“o” and “255” pixels) with the program Image-J in order to count black and white pixels

Measurement of root biomass

In addition to the pixel counts, roots were harvested from each half of the rhizotrons seperately, thoroughly rinsed to remove soil particles and oven dried at 65 °C for 24 h. Dry weight was recorded and the root density in each side was measured as a percentage of the total root density for each rhizotron. The results were then compared with the imaging method based on pixel counts.

Statistical analyses

Differences in shoot biomass as well as in root density in the Ni-spiked zones among all *N. caerulescens* and *S. media* treatments were assessed through one-way ANOVA and Tukey’s HSD post-hoc tests. Welch ANOVA and the Games-Howell post-hoc tests were considered when homogeneity of variance was not met. Differences in root density % between control and Ni-spiked soil zones within each treatment were assessed through Student’s T test for independent samples. Correlations among Ni concentrations in the shoots and root density (pixel counts) in the Ni enriched soil were assessed through the Pearson’s coefficient *r*. All statistical tests were performed with the program IBM SPSS statistics 24 considering a significance level of $p < 0.05$.

Results

Soil characterization

The main characteristics of the control soil, such as pH, pseudo-total and Ni DTPA-extractable concentrations (Zn_{DTPA}, Ni_{DTPA}), Olsen-P, TOC, TN and texture, are shown in Table 1. The control soil was characterized by neutral pH (6.98), high available P (49.3 mg kg^{-1}), high total organic content (8.71 wt%) and total nitrogen (0.50 wt%), sandy loam texture and low pseudo-total (9.36 mg kg^{-1}) and DTPA extractable (0.454 mg kg^{-1}) Ni concentration. Enrichments with the garnierite mineral and Ni-carbonate resulted, as expected, in a total Ni concentration of about 2000 mg kg^{-1} (Table 1). While the addition of garnierite resulted in a negligible pH variation (0.03 units), the Ni-carbonate enrichment caused a pH rise of 0.45 units. Plant available Ni fraction resulted to be low (3.63 mg kg^{-1} Ni_{DTPA}) in the garnierite-spiked soil, while a high Ni availability was measured in the Ni-carbonate treatment (984 mg kg^{-1} Ni_{DTPA}).

Shoot biomass

Values of shoot dry weight are shown in Fig. 3. The Plombieres (Zn) accession of *N. caerulescens* resulted in a higher average biomass per plant (131 mg in the Ni-carbonate treatment; 129 mg in the garnierite treatment) compared to the Bergenbach (Ni) accession (47.7 mg in the Ni-carbonate treatment; 49.1 mg in the garnierite treatment), even if higher biomass values in the Plombieres accessions was statistical significant ($p < 0.05$) only for the Ni-carbonate replicates. The two accessions of *S. media* had comparable biomass (Fig. 3). For both plant species, no significant differences in growth rates were recorded among the two different Ni treatments (Ni-carbonate and garnierite).

Rooting density

For all replicates of *N. caerulescens* and *S. media*, root growth over the transparent surface of the rhizotrons was successfully achieved, allowing for accurate observations and analyses of the root systems. Results of root density performed with the pixel count method and with measurement of root biomass (*mg*) show a very high positive correlation coefficient (Pearson's correlation coefficient $r = 0.93$); From the root density analyses (Figs. 4a and 5a) it can be observed that the Bergenbach (Ni) accession of *N. caerulescens* has a higher percentage of roots in the Ni-spiked soil, compared to the

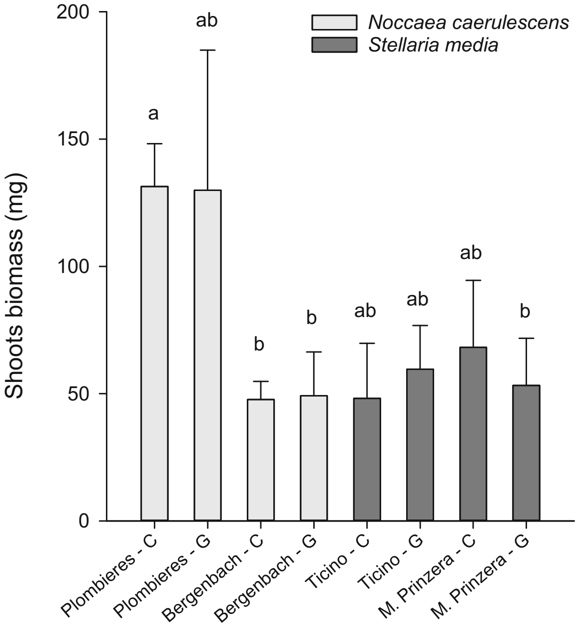

Fig. 3 Shoot dry weight (mg) of *Noccaea caerulescens* and *Stellaria media*. C=Ni carbonate; G = Garnierite. Average values ± standard deviation ($n = 3$). Different letters indicate statistical difference *Welch ANOVA and Games-Howell post hoc test, $p < 0.05$)

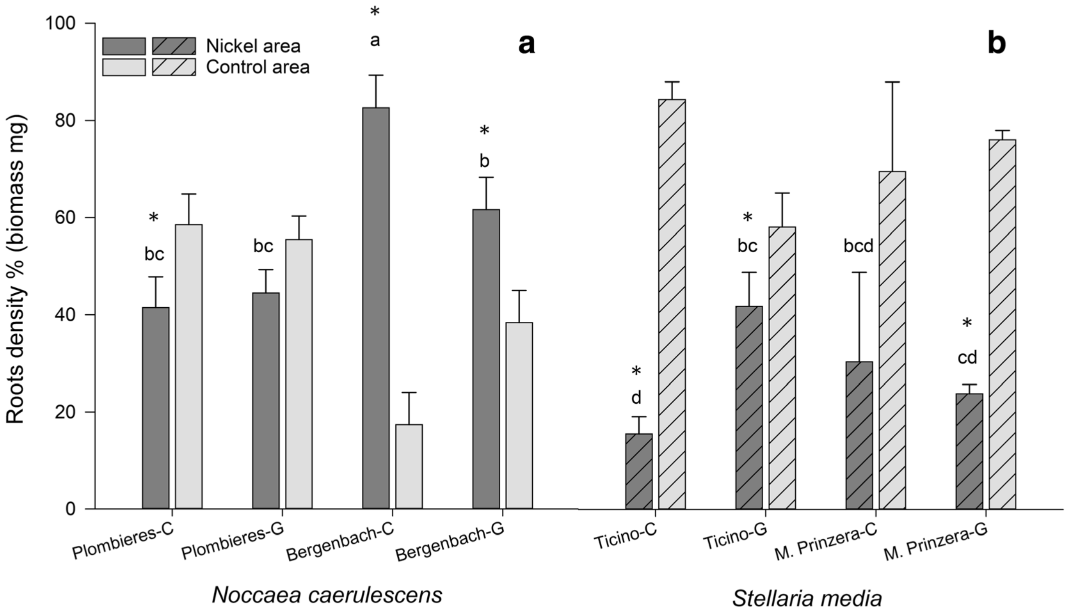

Fig. 4 Root density % in the two areas of the rhizoton (nickel enriched and control) calculated from roots biomass (mg) for **a** *Noccaea caerulescens* and **b** *Stellaria media*. C=Ni carbonate; G = Garnierite. Average values ± standard deviation ($n = 3$). Different letters indicate statistical difference (ANOVA and Turkey's HSD post hoc test. $p < 0.05$) in root density in the nickel enriched area among the different replicates. *indicates statistical difference (Student's T test. $p < 0.05$) between the Ni-enriched and control area within the same replicates

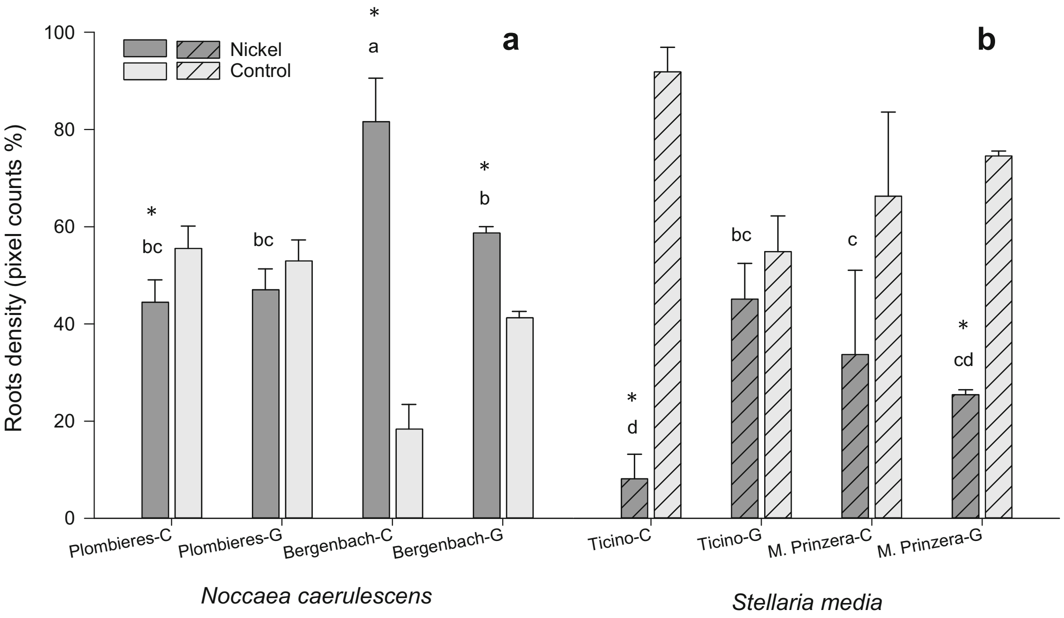

Fig. 5 Root density % in the two areas of the rhizoton (nickel enriched and control) calculated from imaging pixel counts for *Noccaea caerulescens and Stellaria media.* C=Ni carbonate; G = Garnierite. Average values ± standard deviation ($n = 3$). Different letters indicate statistical difference (ANOVA and Turkey's HSD post hoc test. $p < 0.05$) in root density in the nickel enriched area among the different replicates. *indicates statistical difference (Student's T test. $p < 0.05$) between the Ni-enriched and control area within the same replicates

control soil; this trend was more pronounced in presence of Ni-carbonate (roots/Ni side: 81.6% pixel counts; 82.6% root biomass) than in the garnierite treatments (roots/Ni side: 58.7% pixel counts; 61.6% root biomass). Conversely, the Plombieres accession (Zn) had a slightly higher percentage of roots in the control side of the rhizotrons, with lower root density in the Ni-carbonate spiked soil (roots/Ni side: 44.5% pixel counts; 41.5% root biomass), compared to the garnierite (roots/Ni side: 47.0% pixel counts; 44.5% root biomass). Root growth in the Ni-spiked soil was higher in the Bergenbach (Ni) accession compared to the Plombieres (Zn) accession for both Ni forms, even if it was statistically significant only for the Ni carbonate treatment.

In *Stellaria media* (Figs. 4b and 5b), the Ticino accession (non-metalliferous) had a significantly higher percentage of root growth in the control soil for the Ni-carbonate treatment (roots/control: 91.8% pixel counts; 84.4% root biomass); even if not statistically significant for the "pixel count" values (Fig. 5b), a slightly higher root density in the control soil was observed for the garnierite treatment (root/control: 54.8% pixel counts; 58.1% root biomass) compared to the Ni enriched side. The ultramafic accession of *S. media* (Monte Prinzera) also resulted in a higher root density in the control side of the rhizotrons, with higher values (roots/control: 74.5% pixel counts; 76.1% root biomass) in the garnierite treatment than in the Ni-carbonate treatment (roots/control: 66.3% pixel counts; 69.6% root biomass); the difference between root density among control and Ni-spiked soil in the Ni-carbonate treatment was not found to be statistically significant. It can also be observed that the *S. media* Ticino accession had a significantly lower % of roots in the Ni-carbonate spiked soil compared to the Monte Prinzera accession, while an opposite trend is seen in the garnierite treatment (Figs. 4b and 5b).

Shoot concentrations of Ni and other elements

The Ni concentrations in shoots of *N. caerulescens* and *S. media* are shown in Table 2. For both accessions of *N. caerulescens,* a higher Ni uptake in the shoot was recorded in the presence of Ni-carbonate (273 mg kg^{-1} Plombieres accession; 5210 mg kg^{-1} Bergenbach accession) compared to the garnierite-

Table 2 Total concentrations of Ni and other elements in the shoots of *Noccaea caerulescens* and *Stellaria media.*

Plant species	*Noccaea caerulescens*				*Stellaria media*			
Accession	Plombieres		Bergenbach		M. Prinzera		Ticino	
Ni form	Ni-Carbonate	Garnierite	Ni-Carbonate	Garnierite	Ni-Carbonate	Garnierite	Ni-Carbonate	Garnierite
Ca *mg kg*$^{-1}$	8390 ± 27 ***a***	7720 ± 12 ***a***	12,200 ± 20 ***a***	19,000 ± 28 ***a***	6220 ± 8.5 ***e***	4430 ± 4.7 ***f***	4800 ± 13 ***ef***	4060 ± 24 ***f***
Mg *mg kg*$^{-1}$	2480 ± 42 ***a***	3000 ± 33 ***a***	2860 ± 21 ***a***	4200 ± 8.5 ***a***	2520 ± 23 ***e***	1960 ± 1.2 ***e***	2740 ± 10 ***e***	2390 ± 9.8 ***e***
K *mg kg*$^{-1}$	55,000 ± 18 ***b***	47,200 ± 10 ***b***	74,600 ± 30 ***ab***	103,700 ± 12 ***a***	81,600 ± 31 ***ef***	47,400 ± 4.7 ***f***	99,600 ± 19 ***e***	88,200 ± 19 ***ef***
P *mg kg*$^{-1}$	6850 ± 35 ***cb***	5750 ± 11***c***	19,400 ± 43***ab***	32,600 ± 16 ***a***	5920 ± 2.8 ***e***	6150 ± 19 ***ef***	8910 ± 18***f***	7820 ± 15 ***ef***
Ni *mg kg*$^{-1}$	273 ± 38 ***b***	0.771 ± 35***d***	5210 ± 33 ***a***	69.0 ± 51 ***c***	21.5 ± 47 ***e***	< LOQ	8.51 ± 5.1 ***e***	< LOQ
Zn *mg kg*$^{-1}$	1260 ± 56 ***b***	1520 ± 30 ***b***	1410 ± 77 ***b***	4260 ± 22 ***a***	62.9 ± 69 ***e***	48.2 ± 25 ***e***	78.3 ± 24 ***e***	76.5 ± 75 ***e***
Fe *mg kg*$^{-1}$	68.9 ± 27 ***b***	70.3 ± 13 ***b***	137 ± 56 ***ab***	203 ± 6.2 ***a***	73.1 ± 59 ***e***	61.3 ± 17 ***e***	92.5 ± 18 ***e***	77.5 ± 41 ***e***
Mn *mg kg*$^{-1}$	193 ± 54 ***ab***	68 ± 28 ***b***	435 ± 36 ***a***	215 ± 20 ***a***	181 ± 38 ***e***	54.6 ± 8.4***f***	130 ± 15 ***ef***	89.8 ± 39***f***

Data show average values ± relative standard deviation % ($n = 3$). Statistical difference ($p < 0.05$) in element concentrations among accessions and treatments is indicated with different letters. LOQ (limit of quantification) was 0.251 μg kg^{-1}

spiked soil (0.771 mg kg^{-1} Plombieres accession; 69.0 mg kg^{-1} Bergenbach accession), with higher concentrations recorded in the Bergenbach accession compared to the Plombieres accession. For *S. media* a similar trend can be observed (Table 2), as the Ni shoot concentrations are considerably higher in presence of Ni-carbonate. As for *N. caerulescens*, the Ni accession (Monte Prinzera) of *S. media* also resulted in a greater Ni accumulation compared to the non-Ni accession (Ticino), even if no statistical significance was observed. Considering the Ni concentrations in the shoots of *N. caerulescens* and the % of roots growing in the Ni spiked areas (pixel counts), a very strong positive correlation was recorded for the Ni-carbonate (Pearson's r 0.91; p 0.01) and for the garnierite (Pearson's r 0.91; p 0.01) treatments. For *S. media* a very strong positive correlation coefficient (Pearson's r 0.98; p 0.002) is also found between the Ni concentrations in the shoots and the percentage of roots growing in the Ni-carbonate spiked soil. It was not possible to assess correlations (Pearson's r) for the garnierite treatment, since the Ni shoot concentrations were below the limit of quantification (see Table 2).

Considering the Zn shoot concentrations (Table 2), the ultramafic *N. caerulescens* accession (Bergenbach) in the garnierite treatment had a significantly higher Zn accumulation (4260 mg kg^{-1}) compared with the carbonate treatment and with the Plombieres accession for both treatments. No significant difference in Zn shoot concentrations in the *S. media* accession (M. Prinzera) was observed compared with the non-metalliferous accession (Ticino).

The Ca, K and P shoot concentrations were higher in the ultramafic (Bergenbach) accession (Table 2), while Mg concentrations in *N. caerulescens* increased with higher Zn uptake (Pearson's r 0.75; p 0.005).

Discussion

Plant biomass

For both plant species, *N. caerulescens* and *S. media*, no significant differences in plant biomass were recorded within the same accession when growing in the presence of Ni-carbonate or garnierite (Fig. 3), indicating that variations in Ni supply did not influence plant growth. The higher Ni uptake in the shoot (Table 2) observed for the Ni-carbonate treatment in both plant species also did not affect plant biomass production. However, the *N. caerulescens* accession from the Zn-contaminated site (Plombieres) had considerably higher biomass than the ultramafic accession (Bergenbach) (Fig. 3), even if it was not statistically significant for the Plombieres accession growing in the garnierite treatment. Variability in growth rates between the two accessions of

N. caerulescens can be related to the natural morphological and physiological differences in accessions of this species (Ingrouille and Smirnoff 1986; Sterckeman et al. 2017). For example, non-metalliferous accessions of *N. caerulescens* are often characterized by a lower biomass compared to accessions from metal-contaminated soils (Escarré et al. 2013). Comparing a large number of *N. caerulescens* accessions, it was observed that calamine accessions produce the greatest shoot biomass (Sterckeman et al. 2017), as also shown in our experiment. Haines (2002) reported that a higher plant biomass was obtained when roots of *N. caerulescens* were foraging for Zn; in our results, this effect was not observed for Ni, as no biomass variations were recorded in correspondence with higher Ni supply, Ni shoot uptake or root proliferation in Ni-enriched areas (Tables 1 and 2; Figs. 4 and 5). This suggests that, although the root proliferation in Ni-enriched soils is possibly driven by a higher physiological need of this element, high levels of Ni in the shoot are less essential than Zn in terms of plant growth for *N. caerulescens*. Nevertheless, it has to be considered that our experiment was conducted for a time period of 3 weeks, which limits interpretations and comparisons of our results in terms of biomass variations. For *S. media*, no significant differences in growth rates between the two accessions were observed. In this case, root avoidance of the Ni-enriched soil areas might have contributed to reduce toxicity symptoms as, for instance, growth inhibition.

Root foraging

Considering the root density results of our experiment, the method based on pixel counts is a reliable approach for assessing root biomass in rhizotrons, as a very high correlation (Pearson's *r*) was observed between root biomass weight (*mg*) and root density estimated through pixel counts. Therefore, root imaging analysis based on colour contrasts and pixel counts represent a valid and quick method for non-destructive assessments of root density in thin rhizotrons. Further tests should be conducted to define the applicability of this method to larger rhizotrons. The root density analysis (Figs. 4 and 5) has highlighted that the two *N. caerulescens* accessions (Ni accession and Zn accession) are exhibiting different root interactions with Ni enriched areas of the soil. With the more available Ni form (Ni-carbonate), the Plombieres accession (Zn) had a slight avoidance response to Ni, while the Bergenbach (Ni) accession had an explicit preference for Ni enriched soils (Fig. 6). A less pronounced, but similar, response was observed in the presence of the less available Ni form (garnierite).

Our results clearly showed that the root systems of the Ni accession of *N. caerulescens* (Bergenbach) actively proliferate in Ni-enriched soil zones. In a previous

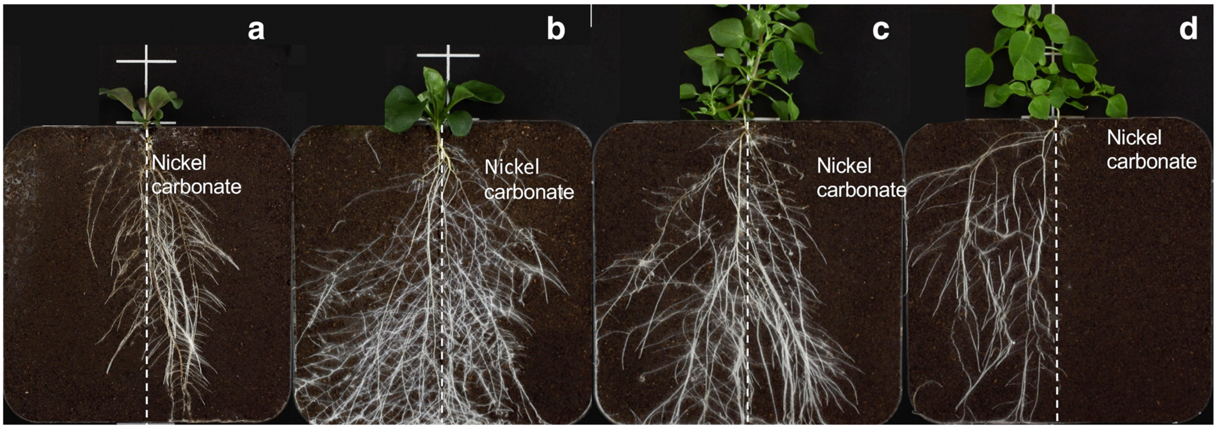

Fig. 6 Rhizotron rooted surfaces at the end of the experiment on the Ni-carbonate treatment with the Ni-spiked soil is on the right half of the rhizotrons: **a** *N. caerulescens* Bergenbach accession; **b** *N. caerulescens* Plombieres accession; **c** *S. media* Monte Prinzera accession and **d** *S. media* Ticino accession

Ni root foraging experiment, no effect on root allocation in *N. caerulescens* in relation to increasing localized total Ni concentrations in the substrate (at 250 and 500 mg Ni kg^{-1}) were reported (Dechamps et al. 2008). Instead, our data showed that the Ni exploration responses in *N. caerulescens* (Bergenbach) was considerably more pronounced in presence of the highly available Ni form (carbonate), suggesting that a higher Ni supply is resulting in an increased root proliferation. The lower Ni concentrations and different Ni forms (Ni (OH)$_2$) tested in Dechamps et al. (2008) could explain these divergent results. Interestingly, *N. caerulescens* (Ni accession) was not only able to tolerate extremely high concentrations of plant-available Ni (Ni$_{DTPA}$ 984 mg kg $^{-1}$) but even to grow most of its roots in this area of the substrate. The less intense root proliferation in the Ni-enriched soil in the garnierite treatment is seemingly due to the lower Ni availability (Ni$_{DTPA}$ 3.63 mg kg^{-1}), which resulted in a weaker response by the root systems. On the contrary, a slight Ni avoidance was recorded in the Plombieres (Zn) accession in the presence of garnierite. Contrasting root response (proliferation vs avoidance) towards Ni of the two *N. caerulescens* accessions suggest that adaptations to different substrate conditions result from different physiological requirements for this metal. While the Plombieres accession appears to tolerate Ni, but to moderately prefer a Ni-free soil, the Bergenbach accession is responding to Ni as if it was an essential nutrient. Increased root growth rate to patches where nutrients are most concentrated is recognized as a common response to nutrient availability (Hutchings and John 2003; Robinson 1994).

In their experiment conducted on *N. caerulescens,* Whiting et al. (2000) observed a root proliferation response to Zn enriched soil patches and suggested that zincophilic accessions of *N. caerulescens* might be actively foraging for Zn in the soil. Haines (2002) also reported a Zn foraging response in *N. caerulescens*, even resulting in higher Zn shoot concentrations for one of the tested accessions (Prayon). Positive root proliferation in Cd-enriched soil patches has also been reported for *N. caerulescens* (Whiting et al. 2000; Schwartz et al. 2003).

The results of Dechamps et al. (2008) showed equal rooting allocation (root biomass) between right and left compartments of the rhizotrons by *N. caerulescens* when growing on uncontaminated or homogeneously metal-spiked substrates, while a higher root allocation of up to 60% by a calamine accession (Prayon) in the Ni enriched compartment (placed either on the right or left side of the rhizotrons) was observed when localized Ni enrichments were tested. Conversely, our results show that the calamine *N. caerulescens* accession (Plombieres) slightly avoids Ni on the right (Ni-rich) rhizotron compartment. Different responses of the Prayon (Dechamps et al. 2008) and Plombieres accessions to Ni could be related to different experimental soil characteristics, for instance, pH values (6.98, see Table 1; 4.65 in Dechamps et al. 2008) or Zn availability, as well as to the Ni forms tested (Ni (OH)$_2$ in Dechamps et al. 2008). In addition, in Dechamps et al. (2008) no pH adjustment has been performed on the "control" rhizotron compartment, where an increase in pH could be expected due to the dissolution of (Ni (OH)$_2$). Possibly, less pronounced Ni foraging responses in Dechamps et al. (2008) compared with the Bergenbach (Ni) accession tested in our experiment can be related to the lower total Ni concentrations in Dechamps et al. (2008) (and presumably to lower Ni availability). Furthermore, the shorter growth period in our experiment (3 weeks) compared to Dechamps et al. (2008) (3 months) seemingly led to clearer observations in terms of foraging responses, as with longer growth times root colonization of the substrate plausibly proceeds in both rhizotrons compartments due to space limitations.

In *S. media*, clear root Ni avoidance was observed in both accessions (Figs. 4b, 5b and 6), with the effect being more evident in the presence of the more available metal form (Ni carbonate). Cadmium avoidance was observed in the non-accumulator *Lupinus albus* (Schwartz et al. 2003), and the same response was reported with the non-accumulators *Thlaspi arvense* in the presence of Zn (Whiting et al. 2000) and *Cicer arietinum* in the presence of Ni (Moradi et al. 2009). To date, no evidence of such root avoidance by metal tolerant plant species have been described for Ni. Being from a non-metalliferous soil, the higher root avoidance of *S. media* Ticino accession in the presence of Ni-carbonate could be expected, while the higher exclusion response of *S. media* from Mt. Prinzera accession to the garnierite mineral cannot be easily explained. It is likely that this accession, adapted to ultramafic conditions, is more efficient at "detecting" Ni than the non-metalliferous accession, thus avoiding Ni-enriched patches more effectively. Our results suggest that metal excluder plant species growing in ultramafic environments might proliferate roots in lower-Ni soil patches and that root avoidance might play a role in their metal tolerance.

Shoot concentrations of Ni and other elements

The higher Ni availability in the Ni carbonate treatment resulted in higher Ni shoot concentrations in both *N. caerulescens* and *S. media* (Table 2). The remarkably higher Ni shoot uptake (Table 2) observed in the Ni accession (Bergenbach) of *N. caerulescens* compared to the non-Ni accession (Plombieres) is again highlighting divergent responses to Ni supply between the two accessions. Only the Ni accession of *N. caerulescens* (Bergenbach) growing in the Ni carbonate treatment resulted in a high Ni shoot concentration (5210 mg Ni kg^{-1}, Table 2) which is higher than the hyperaccumulation threshold of 1000 mg Ni kg^{-1} (Baker et al. 2000; Brooks et al. 1977; Reeves et al. 1996; van der Ent et al. 2013). Literature values from field specimen of the Bergenbach accession (*N. caerulescens*) report shoot Ni concentrations ranging from 396 to 3057 mg kg^{-1} (Chardot et al. 2007), with soil Ni$_{\mathrm{DTPA}}$ concentrations up to about 100 mg kg^{-1}. In our results, a high Ni availability (Ni$_{\mathrm{DTPA}}$; Ni-carbonate treatment) in the substrate resulted in a greater Ni shoot uptake by this accession. As in our experiment, Chardot et al. (2007) also observed that Ni concentrations in the shoot of *N. caerulescens* were significantly correlated with available Ni in the substrate, showing that Ni uptake responds to Ni availability. Dechamps et al. (2008) also reported higher Ni shoot uptake in presence of higher Ni concentrations in the substrate. Other studies on field specimen from Bergenbach (Reeves et al. 2001) reported shoot concentrations of 4625 to 8783 mg Ni kg^{-1}. Surprisingly, in our experiment lower concentrations were achieved, despite the extremely high Ni availability in the substrate (Ni-carbonate treatment); seemingly, the fact that a fraction of the root system was growing on the control soil might have limited the maximum shoot concentrations achieved.

High Zn uptake in the Ni carbonate treatment (Bergenbach accession: 1410 mg Zn kg^{-1}) might also have inhibited Ni accumulation. Also, it has to be considered that a shorter growth period (3 weeks) in our experiment might have contributed to limited Ni uptake compared to field specimens of *N. caerulescens*. In contrast to the Bergenbach accession, *N. caerulescens* from Plombieres had a low Ni accumulation rate (0.771 mg kg^{-1}; Table 2) typical of non-metalliferous plant species when growing in the garnierite treatment. In fact, the leaves of most plant species growing on non-metalliferous soils normally contain Ni concentrations in shoot dry biomass <10 mg kg^{-1} (Reeves et al. 1980). A higher Ni uptake by ultramafic accessions of *N. caerulescens* compared to calamine accessions, has also been validated by genetic studies of Ni tolerance, as described previously (Assunção et al. 2003a). Comparing Cd adaptation mechanisms in two Zn/Cd accessions of *N. caerulescens*, Halimaa et al. (2019) hypothesises that Cd exclusion in the La Calamine accession might play a role in Cd tolerance. A similar exclusion mechanism might explain the lower Ni uptake in the Plombieres accession (which is close to La Calamine). Observing differential root growth in Zn enriches patches, Whiting et al. (2000) suggested that *N. caerulescens* might be capable of zincophilic root foraging responses, but were unable to correlate root proliferation with increased Zn uptake and thus to demonstrate that these were Zn-foraging responses. In our results, the strong positive correlation (Pearson's *r*, $p < 0.05$) observed in both accessions between Ni shoot concentrations and root density in the Ni-enriched zone confirms that *N. caerulescens* is actively foraging for Ni and suggests that a higher uptake of this metal follows a higher physiological requirement.

Reported values of Ni in shoots of *S. media* grown in hydroponic culture range from 166 mg kg^{-1} to 250 mg kg^{-1} (Salinitro et al. 2020). In our experiment, the maximum Ni shoot concentrations in *S. media* were observed, as for *N. caerulescens*, in the Ni-carbonate treatment for the Ni accession of M. Prinzera (21.5 mg Ni kg^{-1}), with lower values for the low Ni accession from Ticino (8.51 mg Ni kg^{-1}). The very strong positive correlation between Ni shoot concentrations and root density in the control soil observed for the Ni-carbonate treatment in *S. media* suggests that rooting Ni avoidance could be a strategy aimed at limiting shoot Ni uptake. According to the definition of excluder plants (Baker 1981), the Ni concentrations in the shoos should remain constant over a range of soil concentrations. Conversely, we observed that higher exposure (through higher root density in the Ni enriched soil) results in a higher Ni concentration in the shoots (Table 2). The fact that with garnierite the shoot Ni concentrations in both *S. media* accessions are non-detectable shows that Ni exclusion effectively limited Ni uptake. Presumably, *S. media* is capable of avoiding Ni enriched soil patches, but when roots are exposed to elevated Ni supply, exclusion mechanisms are limited.

Despite the fact that the highest Zn shoot concentrations in *N. caerulescens* are most frequently observed in calamine accessions (Sterckeman et al. 2017), higher Zn shoot accumulation were reported in non-metalliferous and ultramafic accessions compared to calamine accessions (Escarré et al. 2000, 2013). Dechamps et al. (2008) also report higher Zn uptake in a non-metalliferous accession compared to a calamine accession. Thus, the higher shoot Zn uptake in the ultramafic (Bergenbach) accession (Table 2) is not unexpected. The results of Escarré et al. (2013) suggest that ultramafic accessions of *N. caerulescens* might behave like a non-metalliferous accession, with enhanced capacities of shoot Zn uptake due to low availability in their habitats; this could indeed clarify the higher shoot Zn uptake observed in the ultramafic (Bergenbach) accession in our experiment. On the other hand, the lower Zn uptake in presence of Ni-carbonate could be related to ion competition. Escarré et al. (2013) also observed that Ni addition to a Zn rich soil significantly decreased Zn concentrations in the shoot of *N. caerulescens.* In contrast, a strongly positive correlation between Ni and Zn concentrations in shoots of *N. caerulescens* have been reported (Sterckeman et al. 2017). These contrasting findings (co-transport vs antagonism) may relate to the relative Ni and Zn concentrations tested: while co-transport might occur at balanced and moderate concentrations, antagonism might take place when Zn or Ni are present at high concentrations, as shown in our results (in the Ni carbonate treatment). The lower P, K and Fe shoot concentrations found in our study in the Plombieres accession (Table 2) contradict results of Sterckeman et al. (2017), who reported that calamine accessions contain higher concentrations of these elements in the shoots than ultramafic accessions; only the higher shoot Ca values in the ultramafic (Bergenbach) accessions are in accordance with Sterckeman et al. (2017). These contradicting results could be related to variations in substrates conditions as well as differences in the growing period. Furthermore, the significant positive correlation between Zn and Mg shoot concentrations confirms findings of Dechamps et al. (2005, 2008), who hypothesize the involvement of common transporters for these two elements in *N. caerulescens*.

Conclusions

This study represents the first investigation on root growth responses to artificially Ni enriched soil comparing ultramafic and calamine *N. caerulescens* accessions and differential accessions of a metal tolerant plant species (*S. media*). We have demonstrated that accessions adapted to different substrates conditions exhibit divergent root growth responses in the presence of Ni. Accessions adapted to ultramafic soils appear to have an evident preference for Ni and positive root proliferation in Ni-enriched soils, that consequently enhanced Ni shoot uptake. Thus, we can conclude that a foraging response was observed in the ultramafic accession of *N. caerulescens*, while a slight Ni avoidance was observed in the non-ultramafic accession. In *S. media*, we have described for the first time the root responses of a metal tolerant (excluder) plant species in the presence of soil Ni. Our study suggests that foraging and avoidance of Ni represent different adaptation strategies in ultramafic soils to either support efficient Ni uptake in hyperaccumulators or to assist in metal exclusion in non-accumulating plant species. Further studies should be conducted to better clarify the physiological mechanisms behind these rooting responses to Ni-enriched soils.

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