

Intraspecific variation of nickel and zinc accumulation and tolerance in the hyperaccumulator *Thlaspi caerulescens*

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Abstract Plants from two contrasting populations of the hyperaccumulator *Thlaspi caerulescens*, one from the serpentine area of Monte Prinzerà (MP) in northern Italy and a Belgian calamine population, La Calamine (LC), were crossed to study the genetic correlation of Ni and Zn accumulation as well as Ni accumulation and Ni tolerance. Parental populations and F₃ and F₄ progeny of the interpopulation cross were phenotyped. The phenotype distributions for Zn and Ni accumulation of the parental populations were non-overlapping, with MP having higher foliar metal concentrations than LC. Ni tolerance was also higher in MP, but the parental distributions were overlapping. The F₃ and F₄ progeny exhibited a clear segregation for the Ni and Zn accumulation trait as well as for Ni tolerance. Variance and covariance analysis of the F₃ progeny demonstrated significant heritability values (h²) for Ni and Zn foliar accumulation (0.70 and 0.59, respectively) and Ni tolerance (0.47), as well as a significant positive genetic correlation between the foliar accumulation of Ni and Zn ($r_A^2=0.82$). Ni tolerance and Ni accumulation were uncorrelated. Regressing the F₄ family means

on the F₃ parent values yielded similar estimates for the heritabilities of Ni and Zn accumulation in the leaves (0.66 and 0.55, respectively)

Keywords *Thlaspi caerulescens* · Hyperaccumulation · Tolerance · Heritability · Genetics

Abbreviations

LC La Calamine
MP Monte Prinzerà

Introduction

Heavy metal hyperaccumulation in higher plants is a rare phenomenon, represented by less than 0.2% of all angiosperm species (Baker et al. 2000). Hyperaccumulators (Brookes et al. 1977) are plants able to accumulate metal(s) in their foliage to concentrations of two to three orders of magnitude higher than in normal plants. Enhanced root metal uptake and enhanced root to shoot metal translocation, with a shoot to root metal concentration ratio exceeding unity, are the major characteristics of a hyperaccumulator (McGrath et al. 1993; Pollard et al. 2002). Most of the approximately 400 known hyperaccumulator species are Ni hyperaccumulators, all of which occur on serpentine soils, derived from ultramafic rocks, typically containing 0.1–1% of Ni. Around 15 species are known to hyperaccumulate Zn under natural

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conditions. Both groups include several members of the *Brassicaceae* family, like *Thlaspi* and *Alyssum* species. Some hyperaccumulators are facultative metallophytes, occurring both on metalliferous and non-metalliferous soil, and two of them, *Thlaspi caerulescens* and *Arabidopsis halleri* are more or less widely distributed throughout Europe (Escarré et al. 2000; Bert et al. 2002; Macnair 2002). These species maintain high Zn concentrations in their leaves over a wide range of metal concentrations in the soil (Meerts and Van Isacker 1997; Escarré et al. 2000). When growing in non-metalliferous soil, their foliar Zn concentrations are usually below the arbitrary threshold for hyperaccumulation, but still one order of magnitude higher than in normal plants, at least (Reeves et al. 2001). When grown under controlled conditions at the same Zn supply, non-metallicolous populations often appear to accumulate more Zn than metallicolous ones (Meerts and Van Isacker 1997; Escarré et al. 2000; Schat et al. 2002), showing that these species' apparent ability to accumulate extreme, normally lethal concentrations of Zn in their foliage is constitutive at the species level, although there is a significant variation in degree between populations (Meerts and Van Isacker 1997; Escarré et al. 2000). Both *T. caerulescens* and *A. halleri* can also hyperaccumulate Cd, and serpentine populations of *T. caerulescens* hyperaccumulate Ni (Reeves and Brooks 1983; McGrath et al. 1993; Brown et al. 1995). It is still a matter of debate whether these Cd and Ni hyperaccumulation abilities are also constitutive at the species level. Assunção et al. (2003c) compared *T. caerulescens* populations from different soil types under controlled conditions and showed that some populations, on a total dry weight basis, did not accumulate more Ni or Cd than the non-hyperaccumulating congener *T. arvense*, whereas others hyperaccumulated either Cd or Ni, or both. However, all of the populations showed the strongly enhanced leaf to root metal concentration ratio, both for Ni and Cd, suggesting that enhanced root to shoot translocation of these metals, rather than the enhanced total accumulation as such, represents a constitutive species level trait in *T. caerulescens* (Assunção et al. 2003a, c). Several authors claim that hyperaccumulation is a metal tolerance strategy, and, consequently, that high levels of tolerance to the hyperaccumulated metals are constitutive in hyperaccumulators (Krämer et al. 1997). However, although it is obvious that

hyperaccumulation presupposes tolerance to high leaf-internal concentrations of the naturally hyperaccumulated metals, this does not necessarily mean that hyperaccumulators would inherently tolerate high concentrations of these metals in the soil. For example, Pauwels et al. (2007), using root growth inhibition as a toxicity end point, found significantly higher Zn tolerance in *A. halleri* populations from Zn-toxic, calamine soils than in the non-metallicolous ones. Similarly, using a variety of toxicity end points, metallicolous *T. caerulescens* populations have been shown to be more Zn tolerant than non-metallicolous ones (Assunção et al. 2001; Jiménez-Ambriz et al. 2007). Thus, although there might be a degree of constitutive tolerance, particularly in *A. halleri*, it is evident that calamine populations exhibit enhanced levels of Zn tolerance, compared to non-metallicolous populations. In general, as shown by Assunção et al. (2003c), such enhanced tolerances in metallicolous populations are confined to those metals that are toxically enriched at the populations sites, i.e. Cd and Zn at calamine sites and Ni at serpentine sites, just as in non-hyperaccumulator metallophytes (Schat and Vooijs 1997). Among *T. caerulescens* populations there is a negative, rather than a positive phenotypic correlation between Zn accumulation and tolerance, as found by Assunção et al. (2003b) in a segregating intraspecific cross between metallicolous and non-metallicolous *T. caerulescens*. This suggests that Zn hyperaccumulation, in so far as it segregates in intraspecific *T. caerulescens* crosses is not merely a Zn tolerance strategy. However, one might maintain that, in so far as Zn tolerance and Zn accumulation are constitutive at the species level, they may not segregate in an intraspecific cross, which would obscure any positive genetic correlation. Nevertheless, Macnair et al. (1999) found independent segregation of Zn tolerance and hyperaccumulation of the metal in an interspecific F₂ cross between the metal hyperaccumulator *A. halleri* and the non-hyperaccumulating, non-metallophyte congener *A. lyrata ssp. petraea*, which suggests that the traits are under independent genetic control.

With regard to Cd and Ni, however, the phenotypic correlation between accumulation and tolerance among *T. caerulescens* populations seem to differ from that of Zn. Calamine populations from southern France combine exceptionally high levels of tolerance and Cd accumulation, both in the field and under

controlled conditions (Lombi et al. 2000; Roosens et al. 2003; Zha et al. 2004). Likewise, serpentine populations combine enhanced levels of Ni accumulation and Ni tolerance (Schat et al. 2000). To elucidate the genetic relationship of Ni hyperaccumulation and Ni tolerance in *T. caerulea*, we investigated the co-segregation of these traits in F₃ families derived from a cross between a plant from Belgian calamine population La Calamine (LC), which is lacking Ni hyperaccumulation capacity and one from an Italian serpentine population, Monte Prinzerà (MP), which exhibits high degrees of Ni hyperaccumulation and Ni tolerance (Assunção et al. 2003c). We also established the heritability values for Ni and Zn hyperaccumulation, as well as the genetic correlation between Zn and Ni foliar accumulation rates.

Materials and methods

Plant origin and crossing scheme

A *Thlaspi caerulea* J. Presl and C. Presl plant grown from seeds collected at a strongly Pb/Cd/ Zn-enriched site near La Calamine, Belgium (LC) was crossed to a plant grown from seeds collected at Monte Prinzerà, Italy (MP), an ultramafic site with a high soil Ni concentration. The cross was made by emasculating flower buds of the MP mother plant, followed by repeated hand pollination for 3 days. F₁ seeds were obtained and the seeds of randomly selected F₁ plants were collected after allowing them to self-pollinate. Two F₂ families, numbered 5 and 9, were sown out and 80 plants (61 F₂ (9) and 19 F₂ (5)) were allowed to self-pollinate, resulting in two sets of F₃ families called F₃ (9) and F₃ (5), respectively. Furthermore, phenotyped individuals (see below) of 42 F₃ families (27 F₃(9) and 15 F₃(5)) were randomly selected and allowed to self-pollinate, to investigate the heritability of Ni and Zn accumulation through mid-offspring/parent regression.

Plant culture and vernalisation

Plants were grown from seeds sown on moist peat. Three week old seedlings were transferred to 1 liter polyethylene pots, filled with modified half-strength Hoagland's nutrient solution, containing 3 mM

KNO₃, 2 mM Ca(NO₃)₂, 1 mM NH₄H₂PO₄, 0.5 μM MgSO₄, 1 μM KCL, 25 μM H₃BO₃, 2 μM ZnSO₄, 2 μM MnSO₄, 0.1 μM CuSO₄, 0.1 μM (NH₄)₆Mo₇O₂₄, 20 μM Fe(Na)EDTA. After adding the pH buffer MES in a 2 mM concentration the pH was adjusted to 5.5, using KOH. Pots were randomised within the growth chamber and the nutrient solution was changed twice a week. The crossings and experiments were carried out in a climate chamber (20/15°C day/night; 250 μmoles/m²/s at plant level; 14 h/d; 75% RH). Plants were vernalized at 4/4°C day/night; 200 μmoles/m²/s at plant level; 12 h/day; +/-60% RH for 5–6 weeks in a growth cabinet, while changing the nutrient solution once a week, and then returned to the climate chamber.

Characterization of Ni and Zn accumulation in the F₃ and F₄ families

Three week old seedlings from the parent populations LC and MP (20 LC plants and 20 MP plants), from the F₃ families (1 to 5 plants per family) and from the F₄ families (5 plants per family) were grown in nutrient solution (1 seedling per pot) supplemented with 10 μM NiSO₄ (this concentration was found to yield the highest relative difference in Ni accumulation between LC and MP in previous experiments). The pots were randomised within the growth chamber and the nutrient solution was the same as during preculture and was replaced twice a week. No zinc was added in addition to the standard concentration in the Hoagland solution (2 μM). After 3 weeks of exposure even aged leaves were harvested (3 per plant). The material was dried overnight at 70°C in a stove, followed by digestion in Teflon bombs in a 1:4 mixture of HNO₃ (65%) and HCL (37%) at 140°C for 7 h. The metal compounds were analyzed by flame atomic absorption spectrometry (Perkin Elmer 1100B), and the Ni and Zn concentrations were calculated on a dry weight basis (Table 1).

Ni tolerance assessment

After harvesting the leaves for the determination of Ni and Zn accumulation, the F₃ plants, and those of the parental populations were additionally tested for Ni tolerance, by exposing them to weekly increasing NiSO₄ concentrations [100, 200, 500, 750, 1000 μM], over a time course of 5 weeks. At the end of each

Table 1 Ni and Zn concentration ranges in the foliage of the parental populations and their F₃ progeny

	Number	$\mu\text{g Ni g}^{-1}\text{DW}$	Median	$\mu\text{g Zn g}^{-1}\text{DW}$	Median
F ₃ (9)	133	27–3,733	707	313–5,892	1,380
F ₃ (5)	139	112–4,393	912	822–4,857	1,778
MP	20	1,280–3,215	1,819	3426–9,612	4,786
LC	20	37–108	79	503–1,654	1,148

exposure step, the plants with visible chlorosis were registered and then returned to normal nutrient solution to check the reversibility of the chlorosis. In all cases the chlorosis appeared to be reversed, showing that the chlorosis was induced by excessive Ni exposure.

Statistics

Heritability values for Ni and Zn foliar accumulation and Ni tolerance were calculated from the F₃ data by means of variance partitioning, according to Falconer (1981). The genetic and environmental correlations between the Ni and Zn foliar accumulation rates were calculated from covariance partitioning (Falconer 1981). The heritability values for Ni and Zn foliar accumulation were also calculated through regressing the mid-offspring (F₄) values on the parent (F₃) ones.

The correlation between Ni accumulation and Ni tolerance was analysed by regressing the Ni accumulation rates on tolerance, as well as by a non-parametric 2×2 contingency test, using the median values as class borders.

Results

Ni and Zn accumulation

Foliar Ni accumulation was established in plants exposed to 10 $\mu\text{M NiSO}_4$ in hydroponics. The LC and MP populations exhibited non-overlapping phenotype distributions for Ni accumulation in leaves, with MP accumulating about 25 times more Ni than LC, on average (3.98 to 12.58, and 0.15 to 0.50 $\mu\text{mol/g DW}$, respectively) (Fig. 1). The Zn accumulation distributions of the parent populations were neither overlapping, with MP accumulating about 5 times more Zn than LC, on average (7.94–39.81 $\mu\text{mol Zn/g DW}$ and 1.58–7.94 $\mu\text{mol Zn/g DW}$, respectively) (Fig. 2). The Ni and Zn concentrations in the leaves of the parental populations were significantly correlated ($r=0.691$ and $r=0.574$ for MP and LC, respectively) (Fig. 5). Zn and Ni accumulation clearly segregated in the F₃ population. Both for Ni and Zn accumulation, there was no significant transgression beyond the parental phenotype distributions. There were no

Fig. 1 Frequency distribution over Ni accumulation classes (log scale) among individuals of the parental populations LC (grey bars) and MP (black bars) ($n=40$)

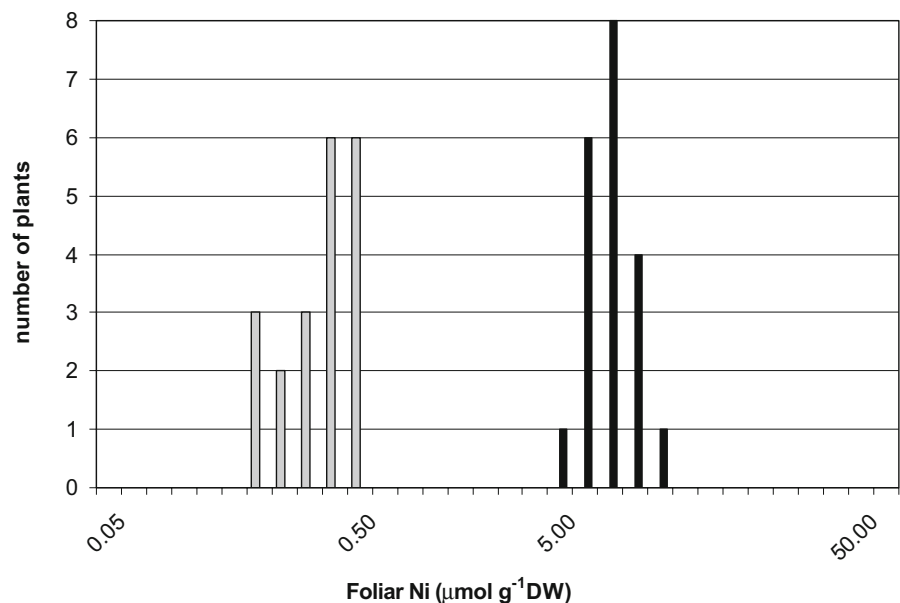
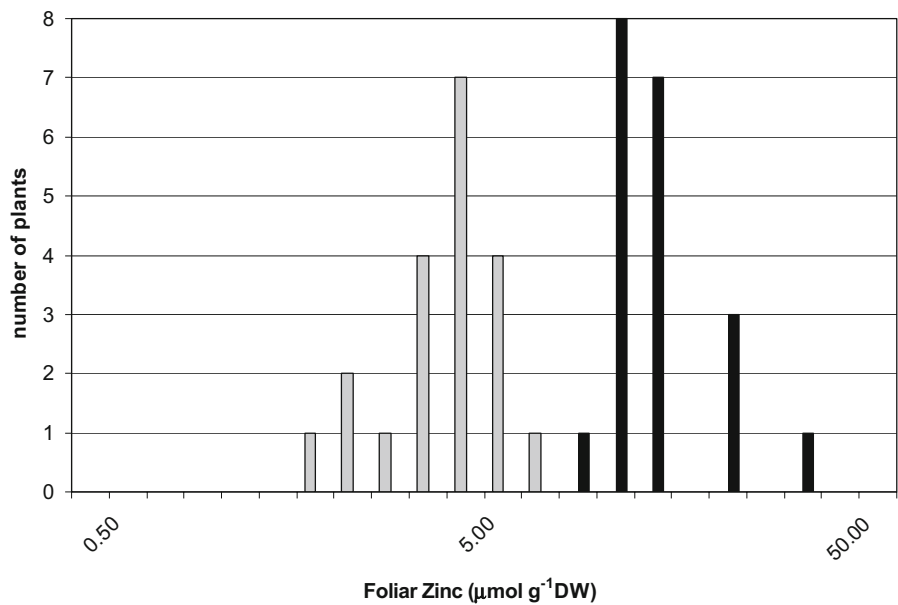


Fig. 2 Frequency distribution over Zn accumulation classes (log scale) among individuals of the parental populations LC (grey bars) and MP (black bars) ($n=40$)



significant differences in leaf Ni and Zn concentrations between $F_3(5)$ and $F_3(9)$ families (Figs. 3 and 4) and therefore both groups were analysed together. Analyses of variance showed significant variation between families ($P<0.001$ and $P<0.01$ for Ni and Zn accumulation, respectively). Also the covariance of Ni and Zn accumulation differed significantly between families ($P<0.001$).

The F_3 progeny showed a significant correlation between Ni and Zn accumulation ($r=0.652$) (Fig. 5). Also the F_4 progeny exhibited a lower but still significant correlation between Ni and Zn accumulation

($r=0.491$) (data not shown). Partitioning of the phenotypic correlation, based on the covariance and variance partitioning, yielded a highly significant positive genetic correlation ($r_A=0.88$) and an insignificant negative environmental correlation ($r_E=-0.11$).

From the variance partitioning of the F_3 data, the heritabilities of the Ni and Zn accumulation phenotypes were calculated as 0.70 and 0.59, respectively. Regressing the mid-offspring values of F_4 families on the corresponding F_3 parent values yielded very similar heritability estimates, 0.66 and 0.58 for Ni and Zn accumulation, respectively ($P<0.01$) (Figs. 6 and 7).

Fig. 3 Frequency distribution over Ni accumulation classes (log scale) of individuals in $F_3(9)$ (grey bars) ($n=133$) and $F_3(5)$ families (black bars) ($n=39$)

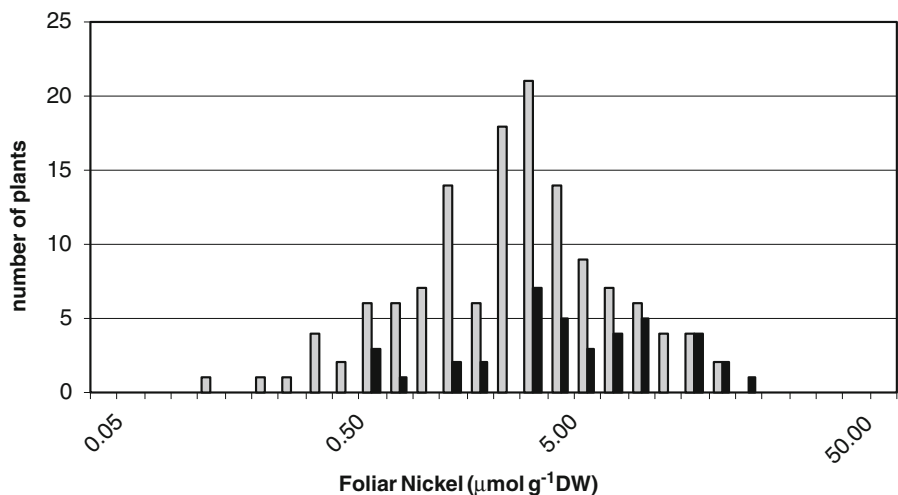
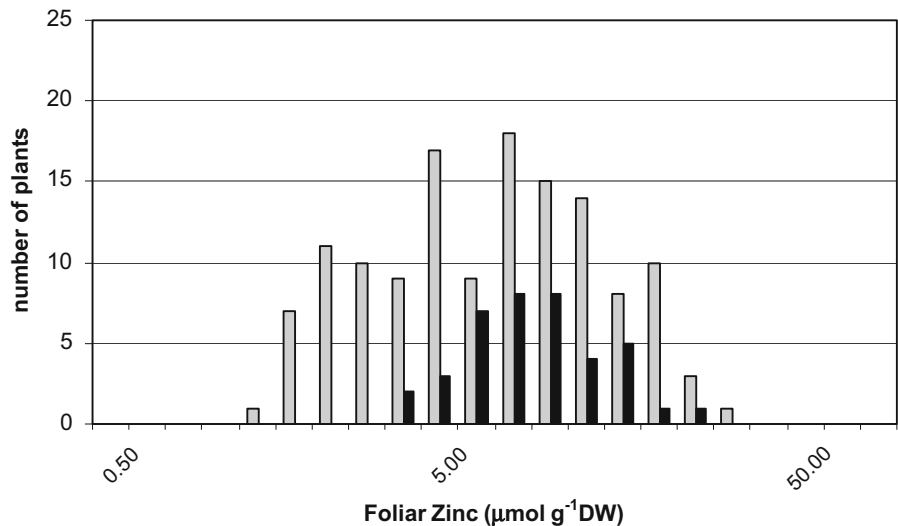


Fig. 4 Frequency distribution over Zn accumulation classes (log scale) of individuals in $F_3(9)$ (grey bars) ($n=133$) and $F_3(5)$ families (black bars) ($n=39$)

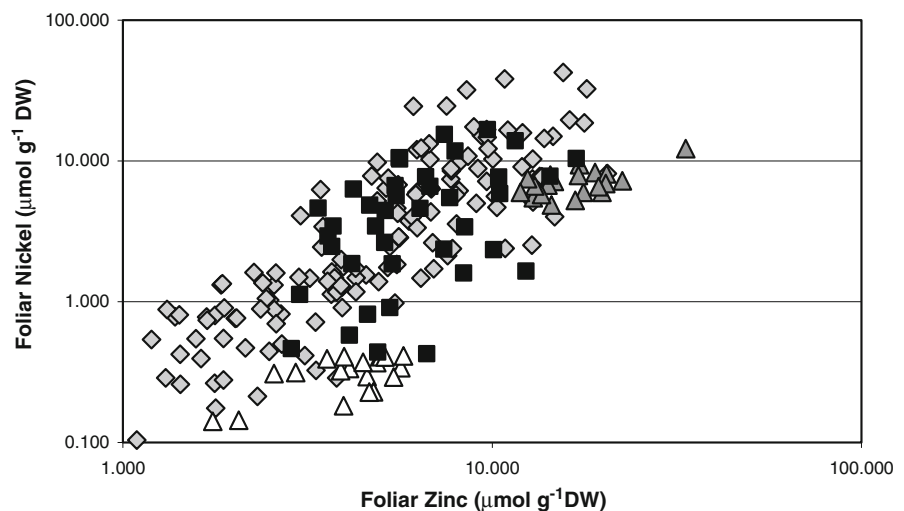


Ni tolerance

After having been phenotyped for Ni and Zn accumulation, plants were exposed to weekly increasing NiSO_4 concentrations, and after each exposure step, the plants with visible chlorosis were registered. Of the parental populations MP showed higher Ni tolerance than LC ($P < 0.01$), on average, although the distributions were broadly overlapping (Table 2). There was a clear segregation of the tolerance trait in the F_3 progeny, and both family sets, $F_3(5)$ and $F_3(9)$, contained individuals that were in the lowest tolerance classes, which were not represented among

MP parent populations, as well as in the highest tolerance class, which was not represented among the LC parent population. Analysis of variance demonstrated significant variation between families ($P < 0.05$) and a heritability value of 0.49. However, there was no correlation between the median Ni concentration in the leaves, measured after 3 weeks of growth at $10 \mu\text{M}$ Ni (see Material and Methods), and the tolerance to this metal (Fig. 8). Also, a 2×2 non-parametric-contingency test with the median tolerance and accumulation values as class borders did not yield any significant association of high accumulation and high tolerance.

Fig. 5 Correlation of Ni and Zn accumulation in the $F_3(5)$, the $F_3(9)$ set of families (black squares, $r=0.66$ and grey squares, $r=0.51$, respectively) ($n=172$) and the parental populations LC (white triangles, $r=0.57$) and MP (grey triangles, $r=0.69$)



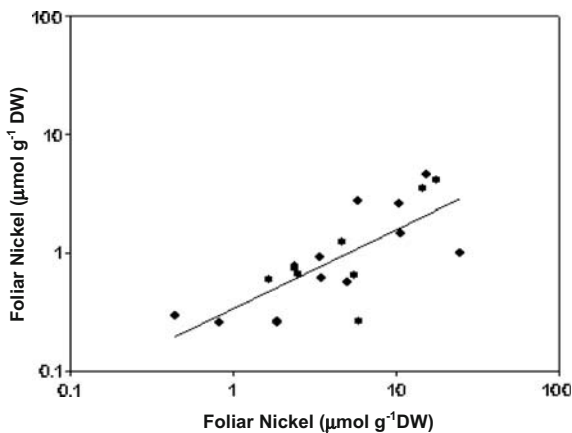


Fig. 6 Mid-offspring/parent regression for Ni concentration in the leaves of F_3 and F_4 progeny ($h^2=0.6668$) ($n=20$)

Discussion

In agreement with a previous study (Assunção et al. 2003a), our results revealed a striking difference between the populations LC and MP with regard to their foliar Ni accumulation capacity. MP plants accumulated, on average, 25 times more Ni in their leaves than LC plants did. Zn accumulation was also higher in MP, but no more than fivefold, on average. Both populations accumulated much more Zn than Ni, although Ni was supplied at a fivefold higher concentration (10 μM Ni versus 2 μM Zn).

The F_3 progeny of the intrapopulation cross showed a broad segregation for foliar Ni and Zn accumulation but significant transgression beyond the

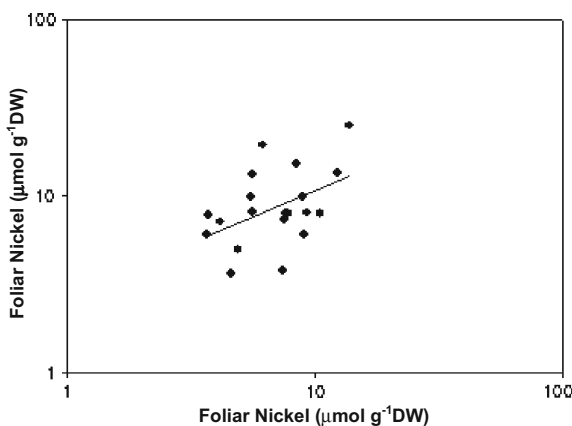


Fig. 7 Mid-offspring/parent regression for Zn concentration in the leaves of F_3 and F_4 progeny ($h^2=0.5870$) ($n=20$)

Table 2 Numbers of Individuals in EC_{100} classes for Ni induced chlorosis for the parental populations LC and MP ($n=70$ per parent) and their F_3 progeny ($n=172$)

	EC_{100} for chlorosis mM Ni in nutrient solution					
	100	200	500	750	1,000	>1,000
MP				31	16	23
LC	3	2	7	52	6	
$F_3(9)$	8	3	8	26	54	36
$F_3(5)$	5		16	4	8	4

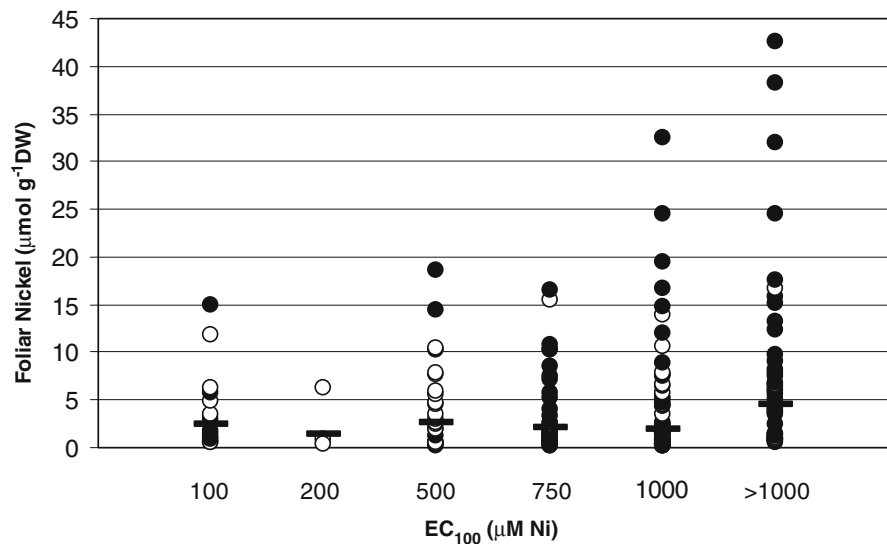
parent phenotypes was not found. Our heritability estimates, 0.70 and 0.59 for Ni and Zn foliar accumulation respectively, demonstrate that the phenotypic variation among the F_3 progeny was mainly attributable to genetic variation.

The segregation patterns in the F_3 progeny, both for Ni and Zn accumulation, were continuous rather than bimodal, with a large fraction of the individuals having Zn and Ni accumulation rates intermediate between those of the parental phenotypes. This may be taken to suggest that the difference in metal accumulation between LC and MP is controlled by more than one gene, and that high accumulation is only partially dominant over low accumulation. However, the heritabilities obtained in this study are too low to exclude the possibility of a single codominant gene.

In previous studies on different intraspecific *T. caerulea* crosses, Assunção et al. (2003b, c, 2006), Zha et al. (2004), and Deniau et al. (2006) obtained evidence of polygenic control of the intraspecific variation in Zn and Cd accumulation, Zha et al. (2004) and Deniau et al. (2006) observed significant transgression for Zn accumulation and Assunção et al. (2006) and Deniau et al. (2006) found that both parents contributed trait enhancing alleles at different loci. The absence of significant transgression in the present study suggests that the trait-enhancing allele or alleles of the genes governing the segregation of Ni and Zn accumulation in the present study were all contributed by the MP parent.

The significant genetic correlation between Zn and Ni accumulation in the F_3 progeny suggests that the superior Ni and Zn accumulation capacities in MP, as compared to LC, are mediated by a combined Zn/Ni accumulation system that is not expressed in LC, at least in part. In agreement with this, Assunção et al.

Fig. 8 Ni accumulation and tolerance of the F₃ progeny (F₃(5) open circles and F₃(9) closed circles). Plants were grown 5 weeks in nutrient solution with weakly increasing Ni concentrations. EC₁₀₀ values correspondent to week number of treatment. Black bars represent the median Ni accumulation



(2001) showed that at equimolar Zn and Ni supply in the nutrient solution, Ni accumulation was strongly inhibited in MP, but not in LC. On the other hand, Zn accumulation was barely affected by equimolar Ni supply, both in MP and LC, suggesting that the Ni hyperaccumulation system in MP has a strong preference for Zn over Ni. Similar results have been obtained for other serpentine Ni-hyperaccumulating *Thlaspi* species (Taylor and Macnair 2006). The tenfold higher foliar concentration of Ni, as compared to that of Zn, found in MP plants growing in their natural habitat, can only be explained by the 50-fold excess of Ni over Zn in the soil at the Monte Prinzera site (Assunção et al. 2003c). Since LC shows far higher shoot to root concentration ratios for both Ni and Zn (Assunção et al. 2003c), it is likely that the higher foliar accumulation of both metals in MP is due to an enhanced capacity for uptake, rather than for root to shoot transport.

In agreement with Assunção et al. (2003a, c), MP was found to be more Ni tolerant than LC, on average. However, the phenotype distributions showed overlap, and were suggestive of intrapopulation variation, although the testing method could have played a role in addition. In any case, there was clearly heritable variation for Ni tolerance in the F₃ progeny. Furthermore, we did not find any significant correlation, nor association, between Ni tolerance and foliar Ni accumulation. It is remarkable however, that virtually all of the highly Ni accumulating plants are

found among the more Ni tolerant ones, although the mean Ni accumulation level does not increase with tolerance (Fig. 6). This might be taken to suggest that high Ni tolerance may be based either on low accumulation or on a mechanism associated with enhanced rather than decreased accumulation. However, the low plant numbers in the low tolerance classes make it difficult to draw any firm conclusion at this point. In different intraspecific *T. caerulescens* crosses there was neither consistent co-segregation of Zn tolerance and Zn accumulation, nor of Cd tolerance and Cd accumulation (Assunção et al. 2003b, c; Zha et al. 2004). Thus, in general, foliar metal hyperaccumulation in *T. caerulescens* doesn't seem to be a strategy for metal tolerance as such.

The evolutionary origin of Ni hyperaccumulation in *T. caerulescens* remains elusive. It is definitely not a constitutive trait, such as in many serpentine *Alyssum* species (Krämer et al. 1996, 2000). It is apparently a low-affinity phenomenon, since it is only possible where the soil Ni availability greatly exceeds that of Zn, i.e. in serpentine soil. In *T. caerulescens*, and possibly in other serpentine *Thlaspi* and *Alyssum* species, the trait could have been evolved through direct selection for Ni accumulation via some pre-existent Zn transporter. Alternatively, selection for Zn accumulation from serpentine soils, which are often poor in Zn, might have produced high degrees of inadvertent Ni accumulation, due to insufficient transporter specificity.

Conclusions

Ni tolerance and Ni accumulation segregated independently in a *T. caerulescens* intraspecific cross, showing that these traits are under independent genetic control. Ni and Zn accumulation were genetically correlated, confirming the hypothesis that Ni hyperaccumulation is accomplished through a Zn accumulation system.

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