

# Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae)

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## SUMMARY

Zinc tolerance was investigated in five populations of *Arabidopsis halleri* (syn.: *Cardaminopsis halleri*) raised from seeds collected from contaminated and uncontaminated sites. Tolerance was measured by determining the concentration which inhibited root growth (EC<sub>100</sub>). *A. halleri* populations from contaminated and uncontaminated sites were found to be Zn-tolerant compared with the Zn-nontolerant species *Arabidopsis thaliana* and *A. lyrata* subsp. *petraea*. At very high Zn concentrations, populations of *A. halleri* from uncontaminated sites were slightly less Zn-tolerant than those from contaminated sites. These observations support the hypothesis that in *A. halleri*, Zn tolerance is largely a constitutive property. One population from an uncontaminated site and one population from a contaminated site were studied for Zn uptake. Zinc content was measured in shoots and roots using a colorimetric test under laboratory conditions. The results showed that whatever their origin, individuals from both populations are Zn accumulators compared with the nonaccumulator species *A. thaliana*. Moreover, the population from the uncontaminated area accumulated Zn in its shoots and roots more quickly than the population from the contaminated site. These results suggest that, in *A. halleri*, Zn accumulation to very high concentration is a constitutive property.

Key words: *Arabidopsis halleri*, zinc, tolerance, accumulation, population differentiation.

## INTRODUCTION

Metal tolerance can be defined as the ability of a plant to survive and reproduce on sites that are toxic or inimical to most other plants because the soil contains elevated concentrations of one or more metals (Macnair & Baker, 1994). This definition of tolerance requires demonstration of the phytotoxicity of the soil and the ability of a plant to grow and reproduce without any sign of toxicity on this phytotoxic soil, where most other plants would stop growing normally and show signs of toxicity (Macnair & Baker, 1994). The existence of plants on contaminated sites raises the question of whether they belong to species of inherent metal tolerance or

whether they are races which have evolved tolerance not possessed by the remainder of the species. Metal tolerance has generally been studied as an example of natural selection in plants, with the evidence that the ability to colonize contaminated areas depends on the ability to evolve ecotypes tolerant to heavy metals. Antonovics *et al.* (1971) state that 'there is no evidence that a species has constitutional tolerance to heavy metals: evolution has always occurred when mine habitats are colonized'. In contrast to this paradigm is the phenomenon of constitutive (= constitutional) metal tolerance, defined as a trait present in all members of populations of a species growing either on contaminated or uncontaminated soils (Baker & Proctor, 1990; Meharg, 1994; Boyd & Martens, 1998). As a result, constitutive tolerance has to be investigated in species which are found on both contaminated and uncontaminated sites

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(pseudometallophytes), where it is possible to differentiate the evolution of ecotypes (population tolerance is correlated with site) from constitutive tolerance (where it is not). There is now evidence that constitutive metal tolerance exists in some species. McNaughton *et al.* (1974), Taylor & Crowder (1984) and Ye *et al.* (1997) compared *Typha latifolia* populations from uncontaminated and contaminated sites and found that, whatever the origin of the population, there is no difference in tolerance. The authors concluded that the species exhibited a constitutive tolerance to Zn, Pb and Cd throughout its range. Constitutive tolerance to Zn was also found in *Thlaspi caerulescens*, in which the metal tolerance of populations from contaminated and uncontaminated sites was compared (Ingrouille & Smirnov, 1986; Lloyd-Thomas, 1995; Meerts & Van Isacker, 1997).

Some metal-tolerant plants are hyperaccumulators (i.e. they are able to accumulate metals in their shoots to an exceptional level (Brooks, 1987)). Most hyperaccumulators are endemic to metalliferous soils, but some have also been described growing on uncontaminated sites. Reeves & Baker (1984) and Boyd & Martens (1998) found, respectively, that the Ni and Zn hyperaccumulator *Thlaspi goesingense* and the Ni hyperaccumulator *Thlaspi montanum* var *montanum*, of which populations from serpentine and nonserpentine soils are described, showed metal hyperaccumulation. Lloyd-Thomas (1995) and Meerts & Van Isacker (1997) found that in the Zn hyperaccumulator *Thlaspi caerulescens*, populations from uncontaminated sites were able to accumulate more Zn than populations from contaminated sites.

*Arabidopsis halleri* (syn: *Cardaminopsis halleri*) is well known for its heavy metal tolerance and hyperaccumulation (Brooks, 1998) and because it is usually described as a plant which belongs to a heavy metal plant-community, e.g. *Armerietum halleri* (Ernst, 1976) or *Holco-Cardaminopsidetum halleri* (Hulbüs, 1981) it is used as an indicator of heavy-metal contamination. It is, indeed, often associated with degraded areas, which contain significantly elevated levels of Zn, Pb and Cd, resulting from human activities such as the metallurgical industry, mining, refuse sites and heavy-metal emissions and is one of the 16 recognized Zn hyperaccumulators, capable of accumulating Zn in its shoots to concentrations of >1% of dry matter (Baker & Brooks, 1989; Brooks, 1998).

A montane species that generally grows at elevations below the timberline, preferably on fresh sandy and oligotrophic soil (Moravec, 1965; Clapham & Akeroyd, 1993), on uncontaminated sites, *A. halleri* belongs to the *Melandrio-Trisetetum* or to the *Cardaminopsidi (halleri)-Agrostitetum* (Moravec, 1965; Ellenberg, 1987). It is distributed throughout Europe except the west, far north, and most of Italy (Clapham & Akeroyd, 1993; O'Kane &

Al-Shehbaz, 1997). Except in France, where it is nonnative and found only on metal-contaminated sites, it grows on both contaminated and uncontaminated sites. Because it occurs both on contaminated and normal soils in the same region, it is considered as a pseudometallophyte (Lambinon & Auquier, 1963; Antonovics *et al.*, 1971). Nevertheless, it is not known whether the distribution of *A. halleri* is related to the ability of its populations to evolve metal tolerance on contaminated sites, or whether this species shows constitutive metal tolerance throughout its range.

All previous studies of *A. halleri* have been of either heavy-metal plant communities (Ernst, 1976; Hulbüs, 1981; Rosenbauer, 1994; Punz, 1995) or, more recently, phytoremediation (Baker *et al.*, 1994; Brooks, 1998; M. J. McLaughlin & R. Henderson, unpublished). To our knowledge, *A. halleri* populations originating from contaminated and uncontaminated sites have never been compared and it is not known whether populations from uncontaminated sites show tolerance to, and the ability to accumulate, Zn. The aim of this study was to investigate these possibilities.

#### MATERIALS AND METHODS

##### *Biology of Arabidopsis halleri*

Recently, *Cardaminopsis halleri* was classified as *Arabidopsis halleri*, based on analyses of rDNA (O'Kane & Al-Shehbaz, 1997). Clapham & Akeroyd (1993) and O'Kane & Al-Shehbaz (1997) divided the species into the subsp. *halleri* and *ovirensis*, based on a number of different morphological traits as well as difference in geographical range. In this study we have followed the O'Kane & Al-Shehbaz nomenclature and the species referred to is the subsp. *halleri*. *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz is a perennial and stoloniferous species. Although there has been no formal study of its mating system, it seems mainly allogamous (M. R. Macnair; V. Bert & P. Saumitou-Laprade, unpublished) and is pollinated by a wide range of insects (unpublished observations). It flowers from April to October.

##### *Collection of seeds and soil samples from field sites*

In July 1997, five populations of *A. halleri* were found in the Czech Republic (Kubova Hut 1, Kubova Hut 2 (both 500–600 m above sea level) and Stary Herstejn (878 m above sea level), and in France (Auby and Courcelles-Les-Lens) (Table 1). Kubova Hut 1 is a meadow beside a railway, where the most abundant plants, apart from *A. halleri*, were: *Deschampsia caespitosa*, *Heracleum sphondylium*, *Lathyrus pratensis* and *Achillea millefolium*. At Kubova Hut 2, *A. halleri* was found alongside a hay meadow, and in Stary Herstejn in a cool, shady

**Table 1.** Details of the sites from which soil samples were collected and *Arabidopsis halleri* populations sampled as seeds

Site	Region	Substratum	Distance between sites (km)	Soil pH	Seed sample size
Kubova Hut 1 (a)	Sumava, Czech Republic	Meadow, shady and moist ditch, alongside the railway	(a-b) = 5 (a-c) = 60	4.8	30
Kubova Hut 2 (b)	Sumava, Czech Republic	Beside road alongside a hay meadow	(b-c) = 65	5.4	18
Stary Herstejn (c)	Sumava, Czech Republic	Slope in a cool, shady wood		5.8	23
Auby (d)	Northern France	Embankment contaminated with Zn, Pb & Cd	(d-e) = 10	5.9	30
Courcelles-Les-Lens (e)	Northern France	Atmospheric dumping of, and embankment contaminated with Zn, Pb & Cd		6.7	7

wood where the most abundant plants, apart from *A. halleri*, were *Mercurialis perennis*, *Galium mollugo*, *Lotus corniculatus* and *Festuca rubra*. In the same season, the two French sites, (Auby and Courcelles-Les-Lens), highly contaminated with Zn, Pb and Cd, were investigated (Table 1). At Auby, an area named le Bois des Asturies served as a waste deposit site for a metallurgical factory (Union Minière). The origin of this population is not well documented. One hypothesis is that *A. halleri* was introduced there 1920–25 as a honey flower (Berton, 1946). A typical heavy-metal plant community colonizes this site, including *Armeria maritima* subsp. *halleri*, *Silene vulgaris* subsp. *humilis* var. *humilis*, *Arrhenatherum elatius*, *Agrostis capillaris*, *Holcus lanatus* and *Festuca rubra* (nomenclature follows Durin *et al.*, 1991). At Courcelles-Les-Lens, the heavy-metal pollution is mainly due to atmospheric deposition from Metaleurop, another metallurgical factory. This site is colonized by *A. halleri*, the grasses *Arrhenatherum elatius* and *Agrostis capillaris*, and *Armeria maritima* subsp. *halleri*.

At each study site, a bulk collection of *A. halleri* seeds was made from 1–5 plants, depending on the number of seeds on each plant. In addition, at three–nine random locations, collections of soil samples were made from around the roots of *A. halleri* plants at depths of 0–20 cm. Two–nine plant samples were also collected for assessment of Zn concentration of leaves.

Seeds of *Arabidopsis lyrata* subsp. *petraea* (O’Kane & Al-Shehbaz, 1997) were collected from Unhošt, uncontaminated woodland in the valley of Loděnice in Central Bohemia (Czech Republic). Seeds of *Arabidopsis thaliana* ecotype Columbia were provided by the Nottingham *Arabidopsis* Stock Centre, Department of Life Science, University of Nottingham, UK. *Arabidopsis thaliana* and *Arabidopsis lyrata* subsp. *petraea* were included in the experiments as Zn-nontolerant and nonaccumulator control species and close phylogenetic relatives of *A. halleri*.

#### Analysis of total and extractable metal concentrations and pH in soil samples

Soil samples were air-dried at 70°C to constant weight. Dried soils were sieved through a 1-mm mesh and ground with a porcelain pestle and mortar.

**Total metals.** Of each prepared soil (three replicates per sample), 0.5 g was cold-digested with HCl and HNO<sub>3</sub> (3:1), for 12 h, then digested at 120°C for 2 h. Acid digests were analysed for Zn, Pb and Cd by flame atomic absorption spectrometry (FAAS).

**Extractable metals.** Of each prepared soil (four replicates per sample), 0.2 g was extracted with 20 ml of ammonium acetate (1 M) in the presence of EDTA (0.01 M) and shaken at 20 ± 1°C for 12 h (pH 7). Concentrations of Zn, Pb and Cd in extractable solutions were analysed by FAAS.

**pH.** Soil pH was measured on 1:1 soil:distilled water mixtures.

#### Plant sample analysis

Plants were rinsed thoroughly in distilled water and dried at 50°C. Of each prepared sample (three replicates per sample), 0.2 g was cold-digested with HNO<sub>3</sub> and HClO<sub>4</sub> (4:1) for 12 h, then digested at 190°C until a final volume of 1 ml was reached. The samples were then diluted to 10 ml with HCl 2M. Concentrations of Zn were analysed by FAAS.

#### Zinc-tolerance experiment

Samples of seeds were taken from each site (Table 1). Two additional species, known to be Zn-nontolerant, were used as controls in the experiment: *A. thaliana* (30 seeds) and *A. lyrata* subsp. *petraea* (30 seeds). Seeds were sown on damp filter paper in a controlled-growth chamber (temperature 24°C day:20°C night; light 16 h day:8 h night) for one month and transferred into 10 µM Zn hydroponic

solution (this concentration allows all plants to grow) with aeration for two further months under laboratory-controlled conditions (temperature 25°C day:15°C night; light 10 h day:14 h night). The nutrient solution used is described in Chaney & Bell (1987): MgSO<sub>4</sub>, 2 mM; Ca(NO<sub>3</sub>)<sub>2</sub>, 0.5 mM; KNO<sub>3</sub>, 0.5 mM; K<sub>2</sub>HPO<sub>4</sub>, 0.1 mM; CuSO<sub>4</sub>, 0.2 μM; MnCl<sub>2</sub>, 2 μM; H<sub>3</sub>BO<sub>3</sub>, 10 μM; MoO<sub>3</sub>, 0.1 μM; FeEDDHA, 10 μM. Zinc was added as ZnSO<sub>4</sub>. Nutrient solutions were changed once a week. The tolerance of the seedlings was determined by the method of Schat & Ten Bookum (1992), which measures the tolerance of a plant by determining the lowest concentration at which no new root growth is produced (= EC<sub>100</sub>). Roots of all seedlings were blackened with activated charcoal and rinsed in deionized water to remove the excess powder. The seedlings were returned to 10 μM Zn for a further week, when roots of the seedlings, with new root growth visible beyond the charcoal-coated roots, were reblackened, and seedlings transferred in successive weeks to 100, 250, 500, 1000 and 2000 μM Zn. At each transfer, those plants which had reached their EC<sub>100</sub> were removed from the experiment and deemed to be nontolerant at this concentration. This use of charcoal does not appear to alter the effective Zn concentration of solutions in these experimental conditions (unpublished results; H. Schat pers. comm.). However, even if the charcoal does affect the interaction of the plants with the Zn in the solution, this cannot affect the relative tolerance of plants as determined by this technique, since all plants will be affected equally.

#### *Zinc-content experiment*

Samples of seeds were taken from Kubova Hut 1 ( $n = 11$ ) and Auby ( $n = 18$ ). *A. thaliana* ( $n = 18$ ) was included in the experiment as a Zn-nonaccumulator control species. Seeds were germinated as before then transferred to a 10 μM Zn hydroponic solution with aeration for two months as in the Zn-tolerance experiment. After two months growing in 10 μM Zn, the shoots and the roots of the seedlings were measured for Zn content. They were then transferred to 50 μM Zn for a further 4 wk with weekly Zn assay. Zinc content of shoots and roots was determined using the colorimetric reagent zincon (Macnair & Smirnoff, 1999). Small leaf samples (10–40 mg) were frozen in liquid nitrogen, extracted with 2% sulphosalicylic acid, and Zn concentration measured at 606 nm on a Shimadzu UV-2401PC spectrophotometer (Shimadzu, Kyoto, Japan) at pH 9.5. Each datum is the mean of three independent samples per plant. Zinc concentrations are expressed as μg g<sup>-1</sup> fresh wt, which would lead to inaccuracy if plants differed substantially in water status. However, all plants were grown in identical conditions in hydroponics, and there was no evidence that the zinc treatments caused systematic variation plant water status.

## RESULTS

### *The concentrations of total and extractable zinc, lead and cadmium in soil samples*

Both total and extractable concentrations of Zn, Pb and Cd were very high in soil samples from Auby and Courcelles-Les-Lens (Table 2). By contrast, in soil samples from Kubova Hut 1, Kubova Hut 2 and Stary Herstejn they were very small (Table 2). As a result, Auby and Courcelles-Les-Lens are considered sites highly contaminated with Zn, Cd and Pb, whereas Kubova Hut 1, Kubova Hut 2 and Stary Herstejn are considered uncontaminated.

### *Zinc concentrations in leaves of wild Arabidopsis halleri plants*

All plant samples, from contaminated and uncontaminated sites, contained elevated concentrations of Zn relative to 'normal' or nonaccumulating plants growing on uncontaminated soils (see \* in Table 2). Plant samples from contaminated sites, Auby and Courcelles-Les-Lens, contained concentrations of Zn higher than 10000 μg g<sup>-1</sup> in dry matter, the threshold of hyperaccumulation for Zn (Brooks, 1998).

### *Assessment of Zn tolerance*

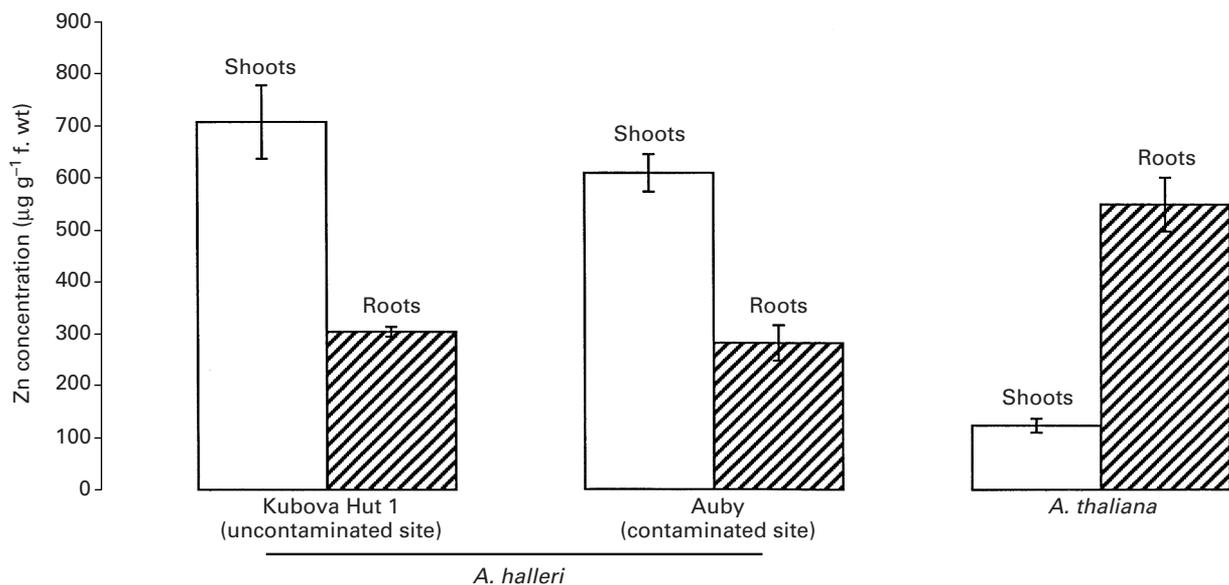
All *A. halleri* seedlings, whatever their origin, showed continued root growth at 100 μM Zn, the first concentration tested and appeared healthy, with no dry or chlorotic leaves. By contrast, the root elongation of all *A. thaliana* and *A. lyrata* subsp. *petraea* seedlings was completely inhibited at 100 μM Zn. Moreover, the seedlings appeared unhealthy, with dry and chlorotic leaves, and no increase in biomass.

At 250 and 500 μM Zn, whatever their origin, all *A. halleri* seedlings produced new roots and looked very healthy (Fig. 1). None of the seedlings had reached their EC<sub>100</sub>. Thus, at these concentrations, there was no difference in the degree of tolerance of *A. halleri* populations. At 1000 μM Zn, some seedlings from contaminated and uncontaminated sites were unable to grow new roots and reached their EC<sub>100</sub>: three seedlings of 18 tested (16.7%) from the uncontaminated site, Kubova Hut 2 and one seedling of 30 tested (3.4%), from the contaminated site, Auby (Fig. 1). At 2000 μM Zn, all populations from both sites contained seedlings which were unable to root (EC<sub>100</sub> reached): Kubova Hut 1, 46.7%; Kubova Hut 2, 27.8%; Stary Herstejn, 30.5%; Auby, 16.7%, Courcelles-Les-Lens, 14.3% (Fig. 1). In order to test whether these proportions differed among populations from contaminated and uncontaminated sites, heterogeneity in proportions of seedlings which had reached their

**Table 2.** Total and extractable concentrations of Zn, Pb and Cd in soil samples where seeds of *Arabidopsis halleri* were collected and Zn leaf concentration in wild plants of *A. halleri* (mean  $\pm$  SE,  $\mu\text{g g}^{-1}$  d. wt;  $n_s$  = number of soil samples per site;  $n_p$  = number of plant samples per site; each sample was assayed by three replicates)

Site	$n_s(n_p)$	Total metals			Extractable metals			Leaf concentration Zn
		Zn	Pb	Cd	Zn	Pb	Cd	
Kubova Hut 1	3(2)	239 $\pm$ 153	20 $\pm$ 9	2 $\pm$ 0.2	185 $\pm$ 120	2 $\pm$ 1.7	0.3 $\pm$ 0.02	3000 $\pm$ 627
Kubova Hut	2(2)	94 $\pm$ 3	16 $\pm$ 6	2 $\pm$ 0.3	3 $\pm$ 0.7	0.5 $\pm$ 0.5	0.6 $\pm$ 0.00	3181 $\pm$ 491
Sary Herstejn	5(2)	143 $\pm$ 25	127 $\pm$ 57	1 $\pm$ 0.5	5 $\pm$ 1	10 $\pm$ 1.6	1 $\pm$ 0.1	3520 $\pm$ 70
Auby	9(9)	48988 $\pm$ 4228	7764 $\pm$ 641	307 $\pm$ 32	5260 $\pm$ 280	1138 $\pm$ 19	88 $\pm$ 1	15707 $\pm$ 1816
Courcelles-Les-Lens	9(9)	4684 $\pm$ 485	5830 $\pm$ 73	165 $\pm$ 1	618 $\pm$ 7	1285 $\pm$ 9	97 $\pm$ 1	12009 $\pm$ 766

\*The mean Zn concentration expected in nonaccumulating plants growing on uncontaminated soil is  $<100 \mu\text{g g}^{-1}$  in dry matter (Brooks, 1998).

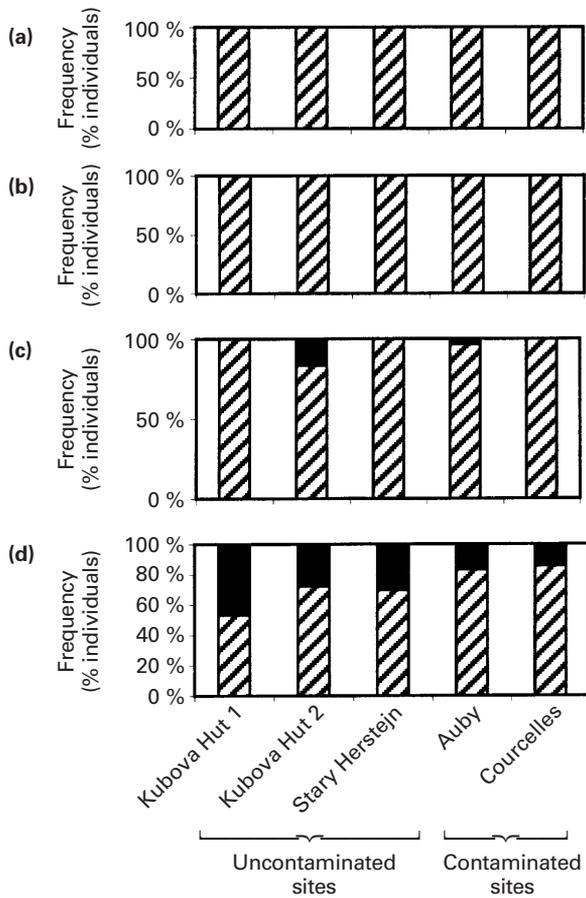


**Fig. 1.** Zinc tolerance in *Arabidopsis halleri* populations from uncontaminated sites (Kubova Hut 1, Kubova Hut 2 and Sary Herstejn) and contaminated sites (Auby and Courcelles-Les-Lens). Tolerance was assessed by exposing seedlings to increasing concentrations of Zn, and determining the concentration at which no new root growth is possible ( $EC_{100}$ ). (a) 250  $\mu\text{M}$ , (b) 500  $\mu\text{M}$ , (c) 1000  $\mu\text{M}$ , (d) 2000  $\mu\text{M}$ . The proportion of seedlings which have ceased rooting (solid bar) or are still rooting (hatched bar) is shown for each population and concentration.

**Table 3.** Analysis of  $G_H$  (Sokal & Rohlf, 1995) for the five populations of *Arabidopsis halleri* shown in Fig. 1, testing whether the proportion of seedlings, which have reached their  $EC_{100}$  and those which have not, is different in all populations ( $ns$ , not significant; \*, significant at  $P = 0.05$ )

Comparison	$G_H$	df	$P$
Overall	7.49	4	0.112 ns
Within populations from contaminated sites	0.02	1	0.887 ns
Within populations from uncontaminated sites	2.48	2	0.289 ns
Between populations from contaminated and uncontaminated sites	4.98	1	0.026*

$EC_{100}$  at 2000  $\mu\text{M}$  was analysed using the  $G$  statistic (Sokal & Rohlf, 1995) (Table 3). Overall comparison showed no significant differences in tolerance level among the five populations, but can be partitioned *a priori* into three orthogonal comparisons: (a) within populations from uncontaminated sites (Kubova Hut 1, Kubova Hut 2 and Sary Herstejn); (b) within populations from contaminated sites (Auby and Courcelles-Les-Lens); (c) between populations from contaminated and uncontaminated sites (Kubova Hut 1, Kubova Hut 2 and Sary Herstejn vs Auby and Courcelles-Les-Lens). The within-group comparisons (a) and (b) were nonsignificant, but a significantly higher proportion of seedlings in the populations from uncontaminated sites reached their  $EC_{100}$  ( $G_H = 4.98$ ,  $df = 1$ ,  $P = 0.026$ ) than in those from contaminated sites.

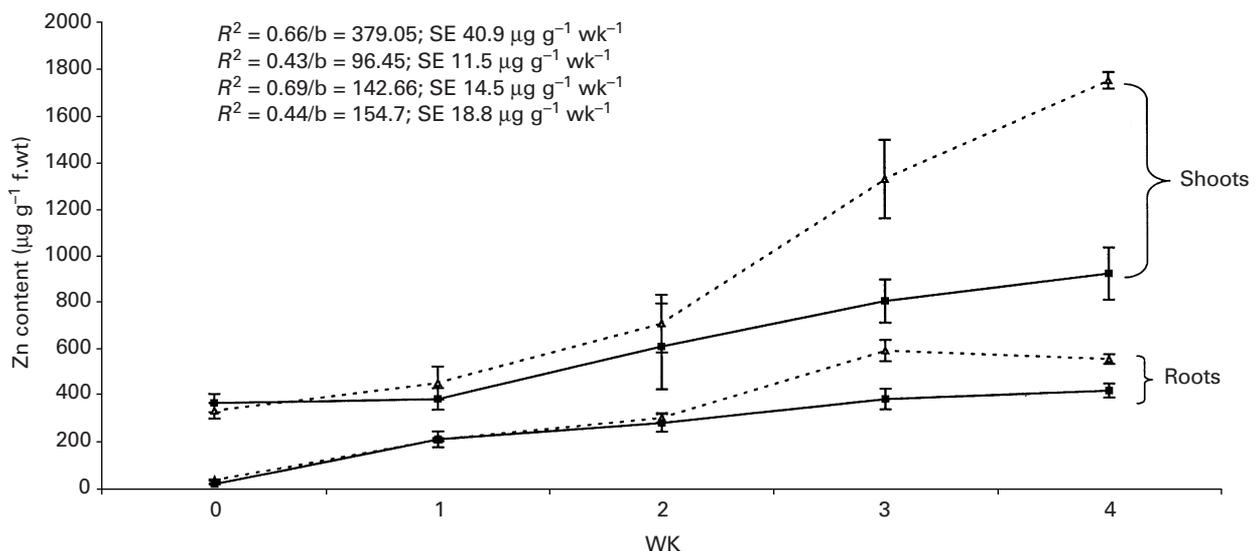


**Fig. 2.** Zinc shoot and root concentrations (mean and SE,  $\mu\text{g g}^{-1}$  f. wt), after 2 wk in  $50 \mu\text{M Zn}$ , of *Arabidopsis halleri* populations from an uncontaminated site (Kubova Hut 1) and a contaminated site (Auby) compared with those of *A. thaliana*. Seedlings were grown in  $10 \mu\text{M Zn}$  hydroponic solution for 1 wk then in  $50 \mu\text{M Zn}$  for 4 wk. The Zn content of shoots and roots was measured weekly.

#### Assessment of zinc content and rate of accumulation

The zinc content of the shoots of seedlings of *A. halleri* from both sites was much higher than that of roots, whereas Zn content of roots of *A. thaliana* seedlings was 4–5 times greater than that of shoots (Fig. 2). Seedlings of *A. halleri*, whatever their origin, showed that most of the Zn was translocated from roots to shoots (shoot:root  $> 2$ ). After 2 wk in  $50 \mu\text{M Zn}$ , *A. halleri* seedlings from uncontaminated (Kubova Hut 1) and contaminated (Auby) sites accumulated the same amount of Zn in their shoots (Student's *t*-test:  $t = 0.77$ ;  $P = 0.45$ ;  $df = 27$ ) and roots (Student's *t*-test:  $t = 0.64$ ;  $P = 0.53$ ;  $df = 27$ ) (Fig. 2).

The rate of accumulation of Zn in shoots and roots of *A. halleri* seedlings from uncontaminated (Kubova Hut 1) and contaminated (Auby) sites was calculated as the regression of concentration on time for 5 wk (1 wk in  $10 \mu\text{M Zn}$  and 4 wk in  $50 \mu\text{M Zn}$ ) (Fig. 3). Regression analysis of shoots and roots of both origins of *A. halleri* seedlings showed that the rate of accumulation increased linearly. Both shoot and root regression coefficients were compared using an F-test. After 5 wk there was a significant difference between both the shoots and the roots of seedlings from the uncontaminated site and those from the contaminated site (shoots:  $F_{1,6} = 32.79$ ,  $P < 0.001$ ; roots:  $F_{1,6} = 5.76$ ,  $P < 0.05$ ). This suggests that the seedlings from the uncontaminated site (Kubova Hut 1) accumulated Zn in their shoots and roots at a higher rate than those from the contaminated site (Auby). Because the rates of accumulation differ, differences between the populations increase with time, accounting for the small



**Fig. 3.** Zinc shoot and root concentrations (mean and SE,  $\mu\text{g g}^{-1}$  f. wt) in *Arabidopsis halleri* populations from an uncontaminated site (Kubova Hut 1, open triangles) and a contaminated site (Auby, solid squares). The seedlings were grown in  $10 \mu\text{M Zn}$  hydroponic solution for 1 wk then in  $50 \mu\text{M Zn}$  for 4 wk. The Zn content of shoots and roots was measured weekly.

difference between the accumulator populations (Kubova Hut 1 and Auby) (Fig. 2) and the much greater difference after 5 wk (Fig. 3).

#### DISCUSSION

##### *Constitutive metal tolerance in Arabidopsis halleri*

We have shown that all populations of *A. halleri* are Zn-tolerant compared with *A. thaliana* and *A. lyrata* subsp. *petraea*. This study is the first to demonstrate high tolerance of Zn in populations of *A. halleri* originating from uncontaminated soils and suggests that a high level of tolerance to Zn ( $> 500 \mu\text{M}$  in hydroponics) is a constitutive trait in *A. halleri*. This is in contrast to the general findings that populations growing on metal-contaminated sites are genetically differentiated (tolerant ecotypes) from populations of the same species growing on uncontaminated sites (Antonovics *et al.*, 1971; Macnair, 1997). Such constitutive tolerance has previously been found in a few other species. In *Typha latifolia*, plants collected from a Zn smelter site did not exhibit higher tolerance than control plants from uncontaminated sites (McNaughton *et al.*, 1974; Taylor & Crowder, 1984). Similarly, *Thlaspi caerulescens*, subsp. *calaminare*, from contaminated soil, and subsp. *caerulescens*, from uncontaminated soil, have shown constitutive tolerance to Zn and Pb (Ingrouille & Smirnov, 1986; Meerts & Van Isacker, 1997). Although all studies of constitutively tolerant species are limited by size of sample population and the incomplete range of distribution of the species from which the populations are taken, the consistent pattern strongly suggests constitutive tolerance in *A. halleri*, *T. caerulescens* and *T. latifolia*.

##### *Degree of tolerance in Arabidopsis halleri populations*

We have found that Zn tolerance is variable between *A. halleri* populations at the highest concentration tested (2000  $\mu\text{M}$ ). The degree of Zn tolerance of seedlings from uncontaminated sites was slightly but significantly lower than that of the seedlings originating from highly contaminated soil. As we have found here in *A. halleri*, *T. caerulescens* showed variation in the degree of tolerance, with subsp. *caerulescens* from uncontaminated sites being less tolerant than subsp. *calaminare* from contaminated sites (Meerts & Van Isacker, 1997). To explain the variation in the degree of tolerance observed between the two *T. caerulescens* subspecies, these authors concluded that the populations growing in contaminated soil might have evolved a higher tolerance which is added to the background level of constitutive tolerance in response to high selective pressure (e.g. exposure to very high level of metals in soils) (Meerts & Van Isacker, 1997). Genetic variation in

the degree of tolerance in species showing ecotypic tolerance is well documented (Macnair *et al.*, 1992; Schat & Ten Bookum, 1992; Schat *et al.*, 1993, 1996; Smith & Macnair, 1998). Smith & Macnair (1998) have shown that in Cu-tolerant *Mimulus guttatus* the variation in Cu tolerance observed among the tolerant populations was due to a variation in modifier genes and not to the major tolerance gene itself. Schat *et al.* (1996) have shown that in *Silene vulgaris* Cu and Zn tolerance were controlled by two additive major genes and modifiers; the most tolerant plants possessed the tolerant genotype for both major genes whereas the least tolerant plants possessed the tolerant genotype for only one. From these results, it can be suggested that all individuals in the constitutively tolerant *A. halleri* or *T. caerulescens* species have the major genes for tolerance, but that the populations growing on contaminated sites have evolved other genes or modifiers to increase tolerance.

##### *Metal accumulation and hyperaccumulation in Arabidopsis halleri*

As expected, the Zn content of shoots of *A. halleri* from the contaminated site was much greater than that of roots (shoot:root ratio  $> 1$ ). This pattern characterizes accumulators (Baker, 1981). Interestingly, *A. halleri* from the uncontaminated site also behaved as a Zn accumulator.

Our results show that the nonmetallicolous population exhibited higher Zn accumulation and transport rate than the metallicolous. Variation in accumulation ability has already been reported in the well known Zn hyperaccumulator *Thlaspi caerulescens* (Lloyd-Thomas, 1995; Meerts & Van Isacker, 1997). Subsp. *caerulescens* from uncontaminated sites accumulated three times more Zn than subsp. *calaminare* from contaminated sites (Meerts & Van Isacker, 1997). Nevertheless, not all hyperaccumulators exhibited variation in accumulation ability. Serpentine and nonserpentine populations of the Ni hyperaccumulators *T. goesingense* and *T. montanum* var. *montanum* were found to accumulate similar and extremely high concentrations of Ni (Reeves & Baker, 1984; Boyd & Martens, 1998).

Our results confirmed the Zn-hyperaccumulator trait of *A. halleri* for metallicolous populations (Brooks, 1998). Interestingly, Zn leaf concentration of nonmetallicolous populations was much higher than that expected for 'normal' plants (e.g. non-hyperaccumulating plants on uncontaminated soil ( $\geq 0.3\%$  (w/w) and  $< 0.01\%$  (w/w) Zn in dry matter, respectively) (Brooks, 1998). Thus, this result, combined with greater accumulation ability of the nonmetallicolous population in hydroponics, allows us to expect Zn hyperaccumulation of such populations if they were transplanted to a contaminated site.

### *Relationship between metal tolerance and accumulation*

Our results showed that metallicolous and non-metallicolous populations differed in Zn tolerance and Zn accumulation. Indeed, the population most tolerant to external Zn concentration is the metallicolous population which exhibited the lowest rate of Zn accumulation and Zn transport. This result is in accordance with that found in *T. caerulescens*, of which metallicolous populations showed a higher Zn tolerance and a lower foliar Zn concentration (Meerts & Van Isacker, 1997). As proposed in *T. caerulescens*, our result suggests that in *A. halleri* the higher tolerance to external Zn in the metallicolous population might be connected with reduced accumulation and/or transport and, thus, accumulation or hyperaccumulation would not be a strategy of tolerance.

### *Origin and evolution of constitutive tolerance and hyperaccumulation*

Our results suggest that in *A. halleri* Zn tolerance and accumulation to very high concentration are constitutive properties. Moreover, we have found that a population from an uncontaminated area is less Zn-tolerant but accumulates more Zn than a population from a contaminated site. From these data, questions can be posed. From where did *A. halleri* arise? Why is *A. halleri* Zn-tolerant and Zn-accumulating on uncontaminated sites? Since *A. halleri* is found on both contaminated and uncontaminated sites, two hypotheses can be suggested. Either *A. halleri* came from an (unknown) metal-contaminated site and has spread from this site to other contaminated and uncontaminated sites (1), or, conversely, it came from an (unknown) uncontaminated site and has spread from this site to other uncontaminated and contaminated sites (2).

Because all populations of *A. halleri* are tolerant, it means that the genes for tolerance *per se* are fixed in the species, and variation in tolerance between populations must be caused by variation in modifier genes (Schat *et al.*, 1993; Smith & Macnair, 1998). Populations from uncontaminated sites were slightly less Zn tolerant than those from contaminated sites. If the first hypothesis were true, this would suggest that some of the modifiers of tolerance have been lost, either through selection, if they have some cost (as postulated by Macnair *et al.*, 1993, for copper-tolerance modifiers), or mutation and drift if they are neutral in an uncontaminated environment. By contrast, hypothesis 2 suggests that the species was pre-adapted to colonize Zn-contaminated sites. Modifiers have then been selected which increased tolerance in populations on contaminated sites. In both cases, tolerance *per se* would appear not to be disadvantageous on uncontaminated sites. Thus this

character has no or low cost and there is no selection against tolerant plants. This might suggest that tolerance in *A. halleri* is a character directly related to a factor other than Zn and thus could be of wider ecological significance. Similarly, the fact that we have found that the population from an uncontaminated site accumulates more Zn than the population from a contaminated site and, much more than nonhyperaccumulating plants on an uncontaminated site, suggests that very high accumulation is not disadvantageous for populations on uncontaminated sites and that this character again might not have evolved for metal accumulation. Similarly, Reeves & Baker (1984), studying *T. goesingense* which possessed a 'constitutional' metal uptake mechanism, concluded that hyperaccumulation was not a microevolutionary response to metalliferous soils. On metalliferous soil, it has been suggested that hyperaccumulation defends Zn and Ni hyperaccumulators against pathogen and herbivore attacks (Boyd & Martens, 1994; Martens & Boyd, 1994; Pollard & Baker, 1997). However, on nonmetalliferous soils, the function of hyperaccumulation is relatively unexplored. Hyperaccumulators accumulate more metals than nonaccumulators on normal soil (Boyd & Martens, 1998; A. J. M. Baker, unpublished), but it has not been shown that this character has any ecological significance. More research is needed on metal content and its consequences for hyperaccumulator species growing on uncontaminated soils, in order to determine which, if either, of the hypotheses postulated by Boyd and Martens (1992) can explain the evolution and maintenance of this character.

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