A broad-scale analysis of population differentiation for Zn tolerance in an emerging model species for tolerance study: *Arabidopsis halleri* (Brassicaceae)

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Abstract

Although current knowledge about the overall distribution of zinc (Zn) tolerance in *Arabidopsis halleri* populations is scarce, the species is an emerging model for the study of heavy metal tolerance in plants. We attempted to improve this knowledge by testing the Zn tolerance of scattered European metallicolous (M) and nonmetallicolous (NM) populations of *A. h.* subsp. *halleri* and *A. h.* subsp. *ovirensis* in hydroponic culture. The occurrence of constitutive tolerance was unconditionally established in *A. h. halleri* and tolerance was extended to the subspecies *ovirensis*. M populations were the most tolerant but there was a continuous range of variation in tolerance from NM to M populations. Finally, relatively high levels of tolerance were detected in some NM populations, suggesting that enhanced tolerance could be present at high frequency in populations that have not experienced metal exposure. We used our results to argue the evolutionary dynamics and origin of Zn tolerance in *A. halleri*.

Introduction

Heavy metal tolerance in plants is commonly defined as the ability to survive on soils which would prove toxic to most living things because they contain (very) high levels of one or more metals (Antonovics *et al.*, 1971; Macnair & Baker, 1994). It was first recognized by Prat (1934) who showed that a copper mine population of *Silene dioica* (syn. *Melandrium silvestre*) was able to grow far better in copper-contaminated soils than populations from uncontaminated areas. Since this initial work, tolerances to a wide range of heavy metals have been encountered in many plant species belonging to widely separated families (Antonovics *et al.*, 1971). These taxa have been classified as either absolute (strict or eu-) or facultative (pseudo-) metallophytes, depending on whether they occur on contaminated sites only or on metalliferous as well as nonmetalliferous soils (Lambinon & Auquier, 1964).

Metal tolerance has been considered to be 'an example of more powerful evolution in action than industrial melanism in moths' (Antonovics *et al.*, 1971). It has therefore been the focus of many studies and the evolutionary framework of heavy metal tolerance in plants is nowadays relatively well documented. Evolutionary studies have argued that metal tolerance could evolve rapidly following exposure to heavy metal stress (Wu *et al.*, 1975; Al-Hiyaly *et al.*, 1988) and that it could have evolved independently in geographically distant conspecific populations (Westerbergh & Saura, 1992; Schat *et al.*, 1996; Vekemans & Lefèbvre, 1997; Koch *et al.*, 1998; Mengoni *et al.*, 2001; Pauwels *et al.*, 2005). Recent genetics studies have demonstrated that in most cases tolerance is governed by a few major genes (for reviews, see Macnair, 1993; Macnair *et al.*, 2000). Quantitative polymorphism is nevertheless usually observed and implies the existence of hypostatic 'modifier' genes that influence the degree of expression of the tolerance phenotype (Schat & Ten Bookum, 1992; Schat *et al.*,

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1993; Smith & Macnair, 1998; van Hoof *et al.*, 2001). In particular, modifiers are supposed to be responsible for the continued evolution towards a high level of average tolerance in metallicolous populations, in response to high levels of metal exposure (Schat *et al.*, 1993; Smith & Macnair, 1998).

In recent years, substantial efforts have been expended to gain further insights in the genetic mechanisms of metal tolerance in plants, i.e. identifying candidate genes and characterizing physiological mechanisms. Thus, many studies have involved high throughput genomics, transcriptomics, proteomics and metabolomics technologies. Genomic and post-genomic technologies are necessarily organized around only a few model species (Feder & Mitchell-Olds, 2003) which makes it crucial to search for the appropriate model. Whereas classical models (e.g. *Arabidopsis thaliana*) were selected by scientists for their particular genetic and development features, the study of ecologically important traits is motivated by additional criteria (Jackson *et al.*, 2002). In the context of heavy metal tolerance in plants, two pseudometallophyte species that both tolerate and hyperaccumulate zinc (Zn) and cadmium (Cd) have recently been proposed: *Arabidopsis halleri* (L.) (O'Kane & Al-Shehbaz) [syn. *Cardaminopsis halleri* (L.) Hayek] and *Thlaspi caerulescens* J. & C. Presl., both Brassicaceae (Assunção *et al.*, 2003b).

Because it is the closest metal tolerant relative of the pre-eminent model system *A. thaliana* (Koch *et al.*, 2001; Al-Shehbaz & O'Kane, 2002), *A. halleri* doubtlessly presents major advantages for the study of tolerance in plants. Indeed, the fact that many molecular tools developed in *A. thaliana* can readily be transferred to *A. halleri* (Mitchell-Olds, 2001; Lexer & Fay, 2005) offers the unparalleled opportunity of an integrative study of metal tolerance. Topics as diverse as the nature, the number and the regulation of genes conferring metal tolerance, the epistatic relationships between them, their origin and evolution of tolerance genes, the impact of selection on these genes can now be addressed. Thus, *A. halleri* is already included in '–omics' studies, and its efficacy in identifying candidate genes involved in metal tolerance and hyperaccumulation has been demonstrated (Becher *et al.*, 2004; Dräger *et al.*, 2004; Weber *et al.*, 2004).

However, the best molecular technologies will be of limited interest if they are not sustained by a good understanding of the ecological range of the model species (i.e. the relative abundance of populations on and off polluted sites) as well as a good understanding of the distribution of tolerance abilities in populations (is this distribution related to the local amount of metals in soils?). So far, in contrast to *T. caerulescens* which has been studied for many decades (Koch *et al.*, 1998; Assunção *et al.*, 2003b), this knowledge remains sparse in *A. halleri* which was sometimes said to be typically found on metalliferous soils (Dahmani-Muller *et al.*, 2000; Pollard

et al., 2002) although numerous NM populations have been mentioned (Bert *et al.*, 2000, 2002). Zn tolerance was assumed to occur throughout the species in *A. halleri* (Bert *et al.*, 2000), but only five populations located in two regions of northern Europe and belonging to the single subspecies *A. h. halleri* were studied in controlled conditions. Meanwhile, the species is widely distributed in Europe and at least two other subspecies are recognized (Al-Shehbaz & O'Kane, 2002). Although it is assumed to be constitutive, Zn tolerance is generally expected to have evolved towards increased levels in M populations of *A. halleri*, in response to high selective pressure caused by high level of metals in soils (Bert *et al.*, 2000). A recent phylogeographic study using cpDNA (Pauwels *et al.*, 2005) showed that *A. h. halleri* population structure was related to geographic isolation rather than to Zn exposure in northern Europe. This implies that geographically close nonmetallicolous (NM) and metallicolous (M) populations (e.g. from southern and northern Germany) are more genetically similar than distant M populations (e.g. from Poland and northern Germany), and that distant M populations have evolved independently. Consequently, if enhanced tolerance was confirmed to be a general feature of M populations, it should have evolved several times in parallel. Moreover, a study of population genetic structure using five nuclear microsatellites over a 500-m-long transect characterized by a gradient of heavy metal concentrations found no evidence of genetic divergence due to spatial heavy metal heterogeneity in these neutral markers, suggesting that long-distance pollen dispersal and extensive pollen flow could attenuate the local effect of metal exposure on differentiation by neutral markers (van Rossum *et al.*, 2004). Altogether, population studies based on neutral genetic markers suggest that the relation between Zn exposure in the wild and the distribution of inherited Zn tolerance abilities in populations might not be as straightforward as usually thought.

The present study attempted to provide a better description and understanding of the distribution of Zn tolerance in *A. halleri* populations, in particular in relation to the available Zn content in soils, and discusses the origin and the evolutionary dynamics of Zn tolerance in *A. halleri* in Europe. To assess accurately the overall level of quantitative polymorphism for Zn tolerance, 28 widely distributed populations of *A. h.* subsp. *halleri*, the only subspecies which has been recorded on metalliferous soils in Europe, were sampled. To be representative of the ecological range of the subspecies, populations were collected in both polluted and nonpolluted areas. In addition, three populations of *A. h.* subsp. *ovirensis* were sampled in the Carpathians Mountains (Romania). To identify the genetic component of population differentiation for Zn tolerance, the tested populations were grown under uniform environmental conditions using hydroponic culture in controlled growth chambers.

Materials and methods

Sampling

In the summers of 1999 and 2003, respectively 12 and 16 widely distributed populations of *A. halleri* subsp. *halleri* were sampled so as to cover the range of the subspecies in continental Europe (Table 1). At each study site, viable mature seeds were collected as maternal progenies from distinct individuals in order to obtain living plants and to perform a tolerance test in hydroponic culture. Soil samples were also collected at each study site in order to estimate the concentrations of extractable Zn. Many results from soil sample analysis have already been published in Bert *et al.* (2002). Additional samples were analysed using the protocol described in Bert *et al.* (2002) and all the results are reported in Table 1.

Most populations (17) were scattered throughout the European mountain ranges (Fig. 1), at middle to high altitudes (mean = 822 m), far from any known metal pollution source, in noncontaminated environments (NM populations, Table 1). They were located in the Tatras and High Tatras mountains (Slovakia), in the Apuseni Mountains (Romania), in the Bohemian Forest (Germany) and on northern and southern slopes of the Alps (Austrian Tyrol; Ticino, Switzerland and Trentino, Italy). Eleven populations (D08–09, D11:13, F01, PL01, PL04, PL06:08, Fig. 1) were sampled outside the mountain ranges, at low to moderate altitudes (mean = 314 m), in the three known polluted areas the species has colonized, i.e. in northern France, Silesia (Poland) and Harz (Germany). In these disturbed regions, *A. h. halleri* populations have been shown to locally cluster into homogeneous genetic assemblages (Pauwels *et al.,* 2005). Since the extension of the *A. h. halleri* distribution area in initially unsuitable disturbed habitats has to be related to anthropogenic metal pollution via industrial or mining activities (Berton, 1946; Fabiszewski, 1986; Ernst, 1990), all populations were classified as M populations and most of them (D12–13, F01, PL04–06–07) indeed occurred on highly polluted soils (Table 1). However, a detailed study of Zn concentration in the soils of each population revealed that the species also colonized slightly polluted habitats (<300 μg g^{-1}, see Bert *et al.,* 2002) where selection pressure towards enhanced tolerance should be strongly reduced (such as in NM populations, see Table 1). In industrial areas, 'non-M' populations (D08-09-11 and PL01–08) may nevertheless differed from true NM ones by their ability to exchange genes with the geographically proximate M populations (van Rossum *et al.,* 2004; Pauwels *et al.,* 2005). Both ecological and genetic considerations (Pauwels *et al.,* 2005) further suggests that M populations might have settled first in these areas and that populations only subsequently colonized slightly polluted habitats. Populations D08-09-11 and PL01–08 were therefore considered to be in a particular category and qualified as NMp (for nonmetallicolous in a polluted area).

In addition to *A. h. halleri* populations, mature seeds from maternal progenies and soil samples from three populations of *A. h.* subsp. *ovirensis* were collected at high altitudes (mean = 2110 m) in the Fagaras Mountains (Southern Carpathians, Romania).

Zinc tolerance experiment

Seeds sampled from each population were first sown in sand filled pots in a greenhouse. After 3 weeks of growth, a maximum of 20 seedlings per population (depending on the germination rate) were transferred into hydroponic conditions in a controlled-growth chamber (temperature 20 °C day : 15 °C night; light 14 h day : 10 h night). Ten-litre containers containing perforated polystyrene trays in which the seedlings were planted into nylon wool were used. In order to avoid local environmental effects, plants were randomly distributed in containers so that each population was represented by at least one individual in each container. Moreover, containers were randomly distributed in the growth chamber and moved around once a week. The composition of the nutrient solution in containers was: 0.2 mM MgSO$_4$, 0.5 mM Ca(NO$_3$)$_2$, 0.5 mM KNO$_3$, 0.1 mM K$_2$HPO$_4$, 0.2 μM CuSO$_4$, 2 μM MnCl$_2$, 10 μM H$_3$BO$_3$, 0.1 μM MoO$_3$, 10 μM FeEDDHA and 1 μM ZnSO$_4$. The nutrient solution was changed once a week. After a 3-week period allowing plants to acclimate to hydroponic conditions, the sequential tolerance test started following the method of Schat and Ten Bookum (Schat & Ten Bookum, 1992). It involves the qualitative measurement of root growth (growth vs. no growth) at sequential concentrations. In our experiment, Zn concentrations varied from 1 μM during the first week to 2000 μM during the ninth and last week of treatment, increasing each week by a constant step of 250 μM. At the start of the sequential test, the roots of all plants were blackened by dipping them in a suspension of activated charcoal, and rinsed in deionized water to eliminate excess of charcoal. Plants were returned to 1 μM Zn for a further week when the presence of any new roots (uncoated) visible beyond the charcoal-coated roots was recorded. Roots were then reblackened and transferred to a fresh test solution with an increased Zn concentration for an additional week. The recording and blackening of new roots were repeated for each plant at each concentration tested. In such a test, the presence of white roots at a given Zn concentration indicates growth. The lowest concentration at which root growth definitively stopped (EC100 in Schat & Ten Bookum, 1992) was determined for each individual. EC100 was interpreted as the lowest concentration that was not tolerated by the individual in question. To check this interpretation, we transplanted 'nontolerant' plants to a fresh solution containing a Zn dose corresponding to their respective EC100s. Mortality usually occurred after

Table 1 Names and locations of population samples of *Arabidopsis halleri*. When shared, the names corresponds to those used in Pauwels *et al.* (2005).

Population	Subspecies	Type	Origin	GPS coordinates North	East	Altitude (m)	Ecological background	N_i est.	Extractable Zn in soil (μM) ± SE (n_s)	Year of test	n
A05	*A. h. halleri*	NM	Tyrol	47°13'778	11°22'779	807	Garden lawn under conifers	<50	7 ± 3 (3)	2004	20
A08	*A. h. halleri*	NM	Tyrol	47°25'177	11°51'957	522	Meadow	100–500	177 ± 123 (4)	2004	20
B01	*A. h. halleri*	NM	Hautes Fâgnes	50°29'63	06°40'	160	Underwood	500–1000	n.a.	2000	16
CH01	*A. h. halleri*	NM	Tessin	45°59'233	08°50'387	340	On a shaded wall, around a private garden	>100	5 (2)	2004	20
CH03	*A. h. halleri*	NM	Tessin	46°10'632	08°43'182	290	Meadow at a wood skirt, along a roadway	<50	17 ± 6 (3)	2004	18
D01 (G1*)	*A. h. halleri*	NM	Bohemian forest	49°10'064	12°09'88	340	Nitrogenous Regen river bank	<50	3 ± 2*	2000	10
D08 (G8*)	*A. h. halleri*	NMp	Harz	51°53'379	10°29'04	190	Old mine (19th century)	<50	123 ± 9*	2000	13
D09 (G9*)	*A. h. halleri*	NMp	Harz	51°53'346	10°25'16	190	Roadside	<50	52*	2000	15
D11 (G11*)	*A. h. halleri*	NMp	Harz	51°51'127	10°21'95	674	Underwood	<50	7*	2000	11
D12 (G12*)	*A. h. halleri*	M	Harz	51°51'191	10°17'790	673	Mine rubble	>500	1272 ± 1047*	2000	16
D13 (G13*)	*A. h. halleri*	M	Harz	51°55'22	10°18'50	237	Roadside and lawn	50–500	405 ± 207*	2000	21
F01 (Auby†)	*A. h. halleri*	M	North	50°25'	03°03'	20	Wood near a smelter plant	>1000	5260 ± 280†	2000/2004	14/20
I01	*A. h. halleri*	NM	Trentin	46°52'783	11°24'308	967	Small meadow along a roadway	<100	13 ± 7 (3)	2004	5
I02	*A. h. halleri*	NM	Trentin	46°49'920	11°43'823	840	Meadow along a footpath	100–500	4 ± 4 (2)	2004	20
I07	*A. h. halleri*	NM	Trentin	46°29'623	10°53'349	1388	Meadow	>1000	8 ± 5 (3)	2004	19
I08	*A. h. halleri*	NM	Trentin	46°29'731	10°53'739	1363	Meadow	500–1000	17 ± 10 (2)	2004	20
I09	*A. h. halleri*	NM	Trentin	46°43'882	11°25'958	1530	Meadow	>5000	11 ± 8 (2)	2004	20
PL01 (P1*)	*A. h. halleri*	NMp	Silesia	50°14'480	18°57'04	200	Wood in Katovice suburbs	>500	22 ± 1*	2000	20
PL04 (P4*)	*A. h. halleri*	M	Silesia	50°29'98	18°55'79	269	Metallurgical factory	>1000	481*	2000	10
PL06 (P5*)	*A. h. halleri*	M	Silesia	50°16'95	19°01'52	306	Old mine	>1000	2490*	2000	10
PL07	*A. h. halleri*	M	Silesia	50°17'014	19°29'564	335	Meadow on a ZN/Pb spoil	n.a.	n.a.	2004	20
PL08	*A. h. halleri*	NMp	Silesia	50°06'243	20°21'569	190	Oak-hornbeam forest	n.a.	n.a.	2004	20
RO05	*A. h. halleri*	NM	Carpathians	46°43'305	23°02'637	990	Footpath along a forest skirt, near a brook	<50	3 ± 3 (2)	2004	20
RO06	*A. h. halleri*	NM	Carpathians	46°39'083	23°02'364	1154	*Picea abies* forest skirt	<50	14 (1)	2004	20
RO08	*A. h. halleri*	NM	Carpathians	46°50'688	22°48'082	900	River bank	<10	3 (1)	2004	20
RO09	*A. h. halleri*	NM	Carpathians	46°49'475	22°45'635	666	Meadow beside a road	<50	9 ± 8 (3)	2004	16
RO14	*A. h. ovirensis*	NM	Fagaras Mountains	45°36'139	24°37'064	2050	Alpine lawn	50–500	n.a.	2004	20
RO15	*A. h. ovirensis*	NM	Fagaras Mountains	45°36'139	24°37'064	2050	Alpine lawn	50–500	n.a.	2004	20
RO18	*A. h. ovirensis*	NM	Fagaras Mountains	45°36'163	24°37'336	2230	Alpine lawn	<50	21 ± 25 (2)	2004	18
SK02 (SI2*)	*A. h. halleri*	NM	Tatras	48°46'17	21°07'81	690	Shady meadow	>500	1*	2000	15
SK05 (SI4*)	*A. h. halleri*	NM	High Tatras	49°16'98	20°09'24	1027	Tatransla javorina (Nature reserve)	>500	1*	2000/2004	21/8

A, Austria; B, Belgium; CH, Switzerland; D, Germany; F, France; I, Italy; PL, Poland; RO, Romania; SK, Slovakia; M, metallicolous populations; NM, nonmetallicolous populations; NMp, nonpolluted populations; ovirensis, *A. h. ovirensis* populations. N_i est.: approximate population size; n_s, number of soil samples; n, sample size for sequential test; n.a., not available.

*Bert *et al.* (2002).

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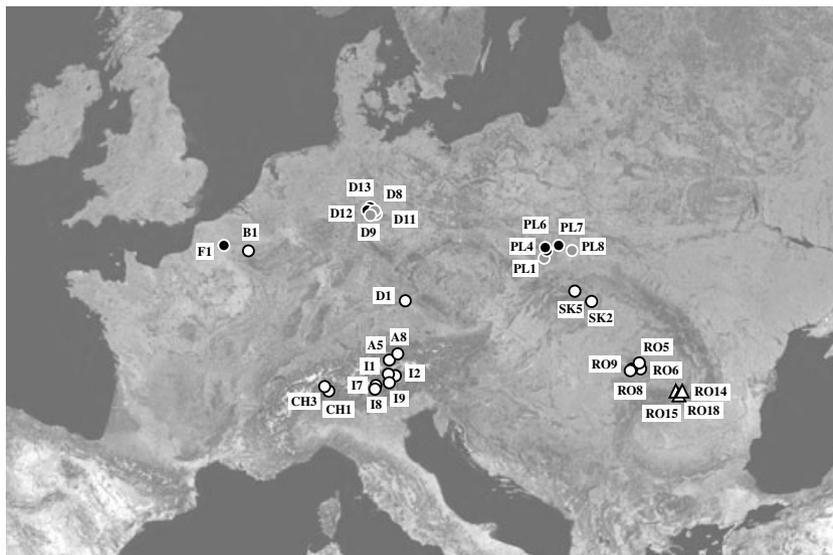
Fig. 1 Distribution map of sampled *Arabidopsis halleri* populations in Europe. Nonmetallicolous, nonmetallicolous in polluted area and metallicolous populations are respectively indicated by white, grey and black circles. Populations of *A. h. ovirensis* are indicated by white triangles.

2 weeks of exposure at such a constant dose, validating the use of 'presence of root growth or not' as an indicator of plant tolerance and, by definition, survival. Plants for which no new roots were observed were removed from the experiment.

Populations sampled in 1999 and 2003 were tested for Zn tolerance separately in time (in spring 2000 and spring 2004, respectively, see Table 1). However, in order to be able to combine survival data from both experiments into a single data set, the same protocol was used, except for the plant density in containers (28 plants per container in 2000, 24 in 2004). Populations F01 (M) and SK05 (NM) were also included in each experiment to allow comparison and to ensure the homogeneity of data sets. In 2000, 12 individuals of *A. thaliana* were randomly distributed in containers so as to be represented one to three times in each container; in 2004, 20 individuals of *A. lyrata petrea* were included and similarly randomized. Both of these nonmetallophyte relatives were used as nontolerant control species.

Statistical analysis

At each experimental dose, root growth was encoded for each plant as a binary variable and interpreted as individual survival or mortality. Survival proportions S_{iX} of individuals from population i that did not reach their EC100 were estimated at each dose X for which an end of growth was observed in that population. Note that the number n_i of survival proportions S_{iX} was a secondary variable that could have differed between populations. The resulting survival curves [$S_i = f(X)$, where 'X' is the concentration of Zn in the test solution] were fitted to sigmoidal dose–response curves with a variable slope by nonlinear regression using GRAPHPAD PRISM version 4.00 for Windows (GraphPad Software, San Diego California

USA, http://www.graphpad.com). The equation used for regression was a two-parameter logistic equation established as following: $S_i = 100/(1 + 10\triangle((\log(T_{50i}) - \ominus \log(X)) * B_i))$ where the variable 'S_i' represents the survival proportions of population i, expressed as a percentage, 'X' is the concentration of Zn in the test solution, expressed in μM, the parameter 'T_{50i}' is the concentration for which 50% of individuals from population i had reached their EC100 (median EC100, $S_{iT_{50i}} = 50$), and the parameter 'B_i' is the slope factor describing the steepness of the curve. Estimated T_{50} values were considered as overall estimations of the tolerance of populations whereas the slope factors B estimated the range of within-population polymorphism for tolerance. For both parameters and for all populations, the standard error (SE) and 95% confidence intervals of best-fit values were estimated by GraphPad prism during curve fitting.

In order to test for differences in average levels of tolerance abilities or within-population polymorphism between groups of *A. h. halleri* populations either tested separately in time (2000 vs. 2004) or corresponding to distinct ecological categories (NM vs. NMp vs. M), box-plots were drawn for both parameters and for each category and Mann–Whitney tests were performed using MINITAB 13.20 (Minitab Inc., State College, PA, USA).

In order to estimate precise confidence intervals for T_{50} and to be able to perform pairwise comparisons of tolerance abilities of populations, the equation used for regression was therefore rewritten so as to directly estimate best-fit values and SE for $\log(T_{50})$ rather than T_{50} (Motulsky 1999) [$S = 100/(1 + 10\triangle((\text{LOGT}_{50} - \log(X)) * B$, where LOGT_{50} and B are the estimated parameters]. LOGT_{50} values were then compared through unplanned comparisons between pairs of populations using the GT2-method for unequal sample size (Sokal & Rohlf, 1981). The method computes comparison
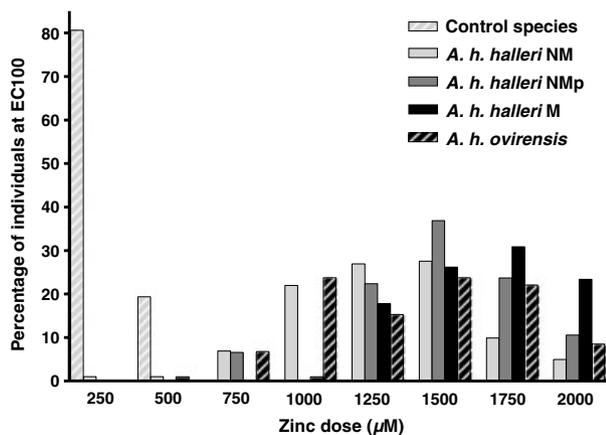
Fig. 2 Histograms showing frequency distribution of EC100s for each category of population. A. h., *Arabidopsis halleri*; M, metallicolous; NM, nonmetallicolous; NMp: nonmetallicolous in polluted area.

intervals for $LOGT_{50}$ for each i population using the two-tailed studentized maximum modulus $m_{z[k*,v]}$ distribution as a critical value (Sokal & Rohlf, 1981). Best-fit values from distinct populations were declared significantly different if their intervals did not overlap. For a 5% experiment-wise error rate, $k* = (35*(35-1))/2 = 595$ and $v = 124$, we used the tabled value $m_{.05[561,120]} = 4.038$ as a conservative approximation to the desired value $m_{.05[595,153]}$.

Results

The distribution of EC100s is presented in Fig. 2. As expected, *A. thaliana* and *A. l. petrea* nontolerant individuals reached their EC100 at first doses of exposure (i.e. 250 and 500 μM) and all *A. halleri* individuals showed clear tolerance abilities. Expect for a few individuals that also reached their EC100 before 750 μM (2% and 1% of NM and M tested individuals, respectively, belonging to populations B1, D1, CH1 and F1), most of the *A. halleri* individuals ranged between 750 and 2000 μM of Zn in solution culture. Interestingly, mortality events were continuously distributed over all doses of exposure, in almost each type of population (i.e. NM, M and ovirensis, the only exception was for NMp populations in which no mortality event was observed at 1000 μM).

All survival data were well fitted by dose–response curves (all $R^2 > 0.93$). Estimated values for both T_{50} and B were precise (relatively low standard errors) and consistent (no aberrant results, Table 2). The number n_i of distinct survival proportion available for curve fitting varied between populations (from $n_i = 3$ to $n_i = 8$, Table 2) and was particularly low for the control species *A. thaliana* and *A. l. petrea* ($n_i = 3$) (Figs 2 and 3). The n_i values of *A. halleri* populations were weakly related to sample size ($R^2 = 0.282$, $P = 0.001$). However, differ-

ences in n_i values did not explain variation in T_{50} and T_{50} standard error ($R^2 = 0.068$, $P = 0.144$ and $R^2 = 0.005$, $P = 0.692$ respectively). A weak but significant correlation was detected between n_i and B ($R^2 = 0.268$, $P = 0.002$), and, to some extent, between n_i and the standard error of B ($R^2 = 0.208$, $P = 0.007$). As a low n_i always meant that the survival proportions dropped from 100% to 0% in a few successive concentrations of exposure, such a correlation was expected. Moreover, differences in B and the standard error of B were not related to differences in sample size ($R^2 = 0.012$, $P = 0.545$ and $R^2 = 0.006$, $P = 0.666$, respectively).

The T_{50} and B values obtained in 2000 and 2004 were very similar for the F01 population and slightly higher for SK05 in 2000 than in 2004 (Table 2). The GT2-test further attested that the $LOGT_{50}$ of both populations did not differ between 2000 and 2004 (see below). Mann–Whitney tests showed that T_{50} values of NM and M *A. halleri* populations obtained from distinct data sets did not differ from each other ($W = 41$, $P = 0.790$ and $W = 18$, $P = 0.561$ respectively). Finally, comparisons of B values and n_i revealed that they did not differ between 2000 and 2004 ($W = 246$, $P = 0.066$ and $W = 307.5$, $P = 0.069$ respectively), in particular when M populations (over-represented in 2000 in comparison with 2004) were removed from the data sets ($W = 112$, $P = 0.317$ and $W = 196$, $P = 0.317$ respectively). These statistical similarities between experiments allowed us to analyse both data sets simultaneously. As both replicates from SK05 and F01 were then considered separately, this led to 33 *A. halleri* populations being tested.

Survival rates of both control species (*A. thaliana* and *A. l. petrea*) severely dropped in the lowest exposure doses (Fig. 3). The respective T_{50} values were low and similar (Table 2). In our experiment, the T_{50} values obtained for control species should be considered to be indicative of the absence of Zn tolerance. In comparison, survival rates remained high to much higher Zn concentrations for all *A. h. halleri* populations (Fig. 3) and T_{50} values were from four to seven times higher than for control species (Table 2). This showed that all *A. h. halleri* populations had a high average level of Zn tolerance, irrespective of their edaphic origin. However, overall differences were observed between NM, NMp and M populations (Fig. 4a). Comparisons of T_{50} revealed that tolerance abilities differed significantly between types (Table 3a). Unilateral tests further showed that T_{50} values were significantly increased from NM populations to M ones, with an intermediate position for NMp populations (results not shown, but see Fig. 4a). Retrospectively, we ensured that such differences remained true in each data set (2000 and 2004) considered separately, at least when NM populations were compared with M ones ($W = 11$, $P = 0.037$ and $W = 112$, $P = 0.032$ respectively). It appeared that the range of T_{50} values was higher for NM populations [mean (m) = 1158.1; standard deviation (SD) = 140.6; coefficient of variation (CV) = 0.12] than for M and NMp ones

Population	n_i	T_{50} (±SE)	$LOGT_{50}$ (±SE)	B (±SE)	d.f.
A05	6	901.9 (±17.71)	2.955 (±8.529×10^{-3})	−6.818 (±0.765)	4
A08	6	1204 (±19.72)	3.081 (±7.113×10^{-3})	−7.218 (±0.805)	4
B01	6	1011 (±52.69)	3.005 (±2.262×10^{-2})	−4.237 (±0.681)	4
CH01	7	1129 (±24.75)	3.053 (±9.523×10^{-3})	−5.868 (±0.724)	5
CH03	5	1099 (±33.02)	3.041 (±1.305×10^{-2})	−7.306 (±1.468)	3
D01	6	1233 (±31.68)	3.091 (±1.115×10^{-2})	−7.533 (±1.347)	4
D08	4	1437 (±21.03)	3.157 (±6.355×10^{-3})	−5.128 (±0.615)	2
D09	5	1335 (±44.04)	3.125 (±1.433×10^{-2})	−7.31 (±1.365)	3
D11	6	1247 (±34.96)	3.096 (±1.217×10^{-2})	−5.727 (±0.865)	4
D12	6	1432 (±35.30)	3.156 (±1.07×10^{-2})	−7.910 (±1.391)	4
D13	5	1384 (±23.06)	3.141 (±7.239×10^{-3})	−8.029 (±1.047)	3
F01–2000	5	1503 (±18.02)	3.177 (±5.205×10^{-3})	−9.909 (±1.083)	3
F01–2004	6	1569 (±39.06)	3.196 (±1.081×10^{-2})	−9.13 (±1.948)	4
I01	4	1092 (±31.44)	3.038 (±1.25×10^{-2})	−4.921 (±0.697)	2
I02	6	993.6 (±12.97)	2.997 (±5.669×10^{-3})	−8.986 (±1.036)	4
I07	6	1348 (±13.99)	3.13 (±4.508×10^{-3})	−8.876 (±0.720)	4
I08	5	1144 (±27.37)	3.058 (±1.039×10^{-2})	−7.451 (±1.276)	3
I09	7	1007 (±23.59)	3.003 (±1.017×10^{-2})	−5.516 (±0.629)	5
PL01	6	1407 (±16.84)	3.148 (±5.196×10^{-3})	−9.078 (±0.912)	4
PL04	5	1509 (±24.02)	3.174 (±1.299×10^{-2})	−5.827 (±0.742)	3
PL06	4	1648 (±15.13)	3.217 (±03.99×10^{-3})	−15.58 (±1.778)	2
PL07	6	1495 (±38.91)	3.175 (±1.13×10^{-2})	−8.115 (±1.574)	4
PL08	7	1390 (±22.05)	3.143 (±06.89×10^{-3})	−7.324 (±0.768)	5
RO05	5	1262 (±66.1)	3.101 (±2.275×10^{-2})	−5.886 (±1.607)	3
RO06	8	1208 (±42.52)	3.082 (±1.528×10^{-2})	−6.06 (±0.451)	6
RO08	6	1199 (±13.01)	3.079 (±4.714×10^{-3})	−4.375 (±0.923)	4
RO09	6	1377 (±49.84)	3.139 (±1.572×10^{-2})	−6.796 (±2.316)	4
RO14	6	1368 (±65.99)	3.136 (±2.096×10^{-2})	−5.942 (±1.105)	4
RO15	6	1162 (±15.05)	3.065 (±5.627×10^{-3})	−7.092 (±0.495)	4
RO18	7	1170 (±59.53)	3.068 (±2.21×10^{-2})	−6.045 (±1.379)	5
SK02	5	1022 (±51.61)	3.01 (±2.192×10^{-2})	−5.136 (±0.897)	3
SK05–2000	5	1408 (±35.23)	3.149 (±1.087×10^{-2})	−11.16 (±2.746)	3
SK05–2004	5	1208 (±28.06)	3.082 (±1.009×10^{-2})	−7.165 (±1.18)	3
A. thaliana	3	245.8 (±0.133)	2.389 (±1.492×10^{-4})	−33.24 (±1.072)	1
A. l. petrea	3	232.4 (±1.175)	2.36 (±2.229×10^{-3})	−30.13 (±1.272)	1

Table 2 Best-fit values and standard errors of T_{50}, $LOGT_{50}$ and B.

Nonmetallicolous, nonmetallicolous in polluted area and metallicolous populations are respectively indicated in light grey, dark grey and black.

n_i, number of doses for which an end-of-growth event was observed in the population i, corresponds to the number of survival proportions available for survival curves fitting; d.f., degree of freedom of parameters (note that d.f. are equal to $n_i − 2$ rather than $n_i − 1$ because both parameters were estimated at the same time, Motulsky, 1999); SE, standard error.

(m = 1503.4; SD = 86.4; CV = 0.06 and m = 1363.2; SD = 74,8; CV = 0.06 respectively). This was confirmed by a Levene test of equality of variances performed using MINITAB 13.20 (data not shown).

Comparisons of $LOGT_{50}$ values confirmed that the control species did not differ from each other and that the Zn tolerance of all *A. h. halleri* populations tested was considerably higher (Fig. 5). The trend towards enhanced tolerance in M populations was also confirmed, with most M populations statistically differing from seven NM populations (out of 18). In particular, the tolerance of the PL06 population was statistically higher than the tolerance of most NM populations (16 of 18). However, twenty-one *A. h. halleri* populations (M, NM or NMp) could not be distinguished from each other. This demonstrated that, rather than discrete statistical categories, a continuum existed from the least tolerant population (A05, NM) to the most tolerant one (PL06, M). Finally, the test showed that the more tolerant NM populations (I07 and RO09) differed significantly from the less tolerant ones (from A05 to CH03), thus confirming a higher heterogeneity of tolerance abilities in this group.

Comparisons of slope factors in *A. h. halleri* revealed that M populations had significantly higher slope factors than NM populations (Table 3b). Because of the wide range of values (Fig. 4b), NMp populations did not differ either from M or NM populations (although a unilateral test showed that NMp populations had higher B values than NM ones, $W = 84$, $P = 0.04$). This suggested an overall tendency towards a reduction of polymorphism
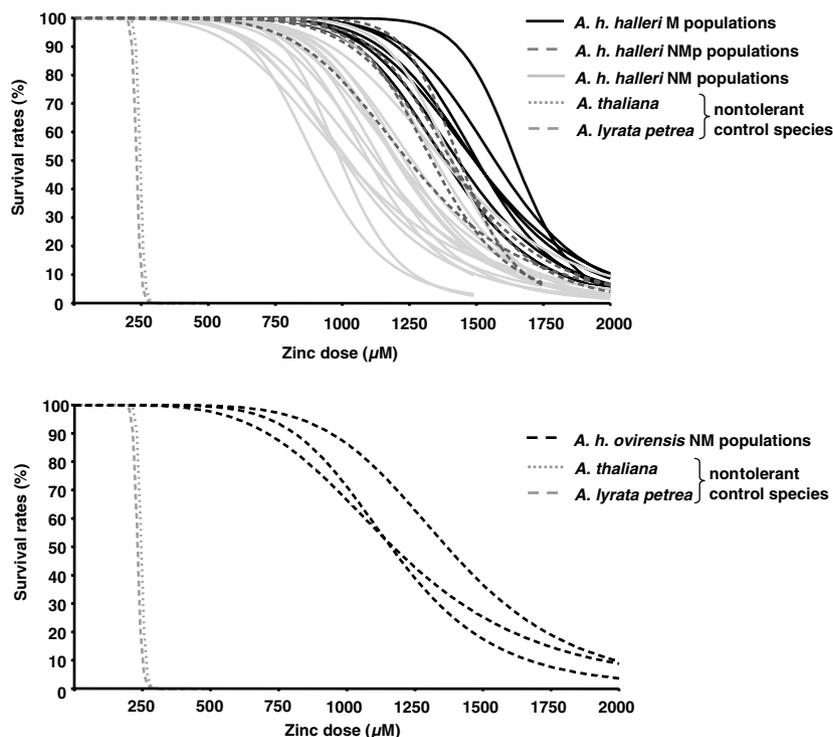
Fig. 3 Population survival curves obtained from fitting to a sigmoidal dose–response model. A. h.: *Arabidopsis halleri*.

around the median EC100 in populations occurring in polluted areas.

In comparison with *A. h. halleri*, populations of the *A. h. ovirensis* subspecies showed moderate to average tolerance abilities (Table 2). Tolerance in these populations was intermediate between NM and NMp *A. h. halleri* populations (Fig. 4a) from which they did not differ statistically (Table 3a). This was confirmed by the *post hoc* comparisons of $LOGT_{50}$ that revealed that *A. h. ovirensis* populations did not differ from most of the *A. h. halleri* populations but did from the two extremes (A05 and PL06, see Fig. 5). The levels of polymorphism in the *A. h. ovirensis* populations tested were more similar to NM populations of *A. h. halleri* (Fig. 4b) and differed significantly from M populations (Table 3b).

Discussion

Constitutive zinc tolerance in the *A. halleri* population in Europe

Our survey confirmed that *A. halleri* is clearly pseudometallophyte in Europe. Most of its populations occurred in mountain areas, at medium to high altitudes, on soils with low levels of metal content. In contrast, in our sampling, both M and NMp *A. h. halleri* populations are not distributed throughout the species range but at the northern margin of the species range. However, most if not all individual we tested showed tolerance abilities and each population of *A. halleri* we tested showed high

average levels of Zn tolerance, independently of the edaphic or geographic origin. We thus largely extended the results obtained from two M populations from northern France and three NM populations from Czech Republic by Bert *et al.* (2000) and strongly established that Zn tolerance is actually constitutive in *A. h.* subsp. *halleri*. Nevertheless, in a few populations, in particular in NM populations, survival rates fell slightly in the first exposure doses, suggesting that rare genotypes with low tolerance abilities may occur. Such an early stop in rooting could also be an artefact of the sequential test and would have to be confirmed by measurement of the heritability of the trait. An appropriate screening of within-population polymorphism using a constant Zn concentration (e.g. 250 μM) could be helpful in identifying putative nontolerant or low tolerant genotypes that would prove particularly useful in genetic studies of metal tolerance mechanisms (Macnair *et al.*, 1999; Bert *et al.*, 2003, G. Willems, C. Godé, D.B. Dräger, M. Courbot, N. Verbruggen, P. Saumitou-Laprade, unpublished data).

Zinc tolerance was not only shown to be constitutive in *A. h.* subsp. *halleri* but also extended to *A. h.* subsp. *ovirensis*. Although no current local heavy metal exposure was detected, we showed Zn tolerance and within-population polymorphism in *A. h. ovirensis* populations. Average tolerance of *A. h. ovirensis* populations was quite similar to those of *A. h. halleri* NM populations, i.e. populations that had definitely not been exposed to high levels of heavy metals in the recent times. Further studies should verify if Zn tolerance is not only constitutive in
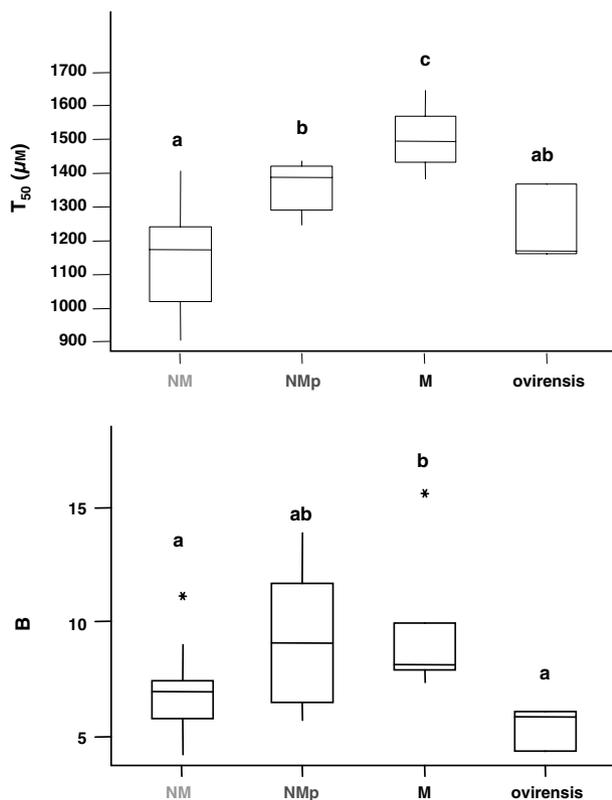
Fig. 4 Box plots of T_{50} and B obtained with MINITAB 13.20 on the entire data set. Different letters above the box plots indicate a significant difference at the 5% level. M, metallicolous populations; NM, nonmetallicolous populations; NMp, nonmetallicolous populations in polluted area; ovirensis: *A. h. ovirensis* populations; *outlier (i.e. any data that is distant from the upper or lower quartile by more than 1.5 times the standard error).

*A. h.* subsp. *halleri* but also species-wide in Europe, i.e. constitutive in both subspecies.

Origin of constitutive zinc tolerance in *A. halleri*

Constitutive metal tolerance is a rare phenomenon in pseudometallophytes (for a review, see Pollard *et al.*, 2002). The most parsimonious hypothesis for the origin of constitutive tolerance would assume that it has been acquired only once. Given the very likely recent history of M populations, their marginal position and the extreme conditions they encountered on polluted soils, it is highly unlikely that metal tolerance spread from an initial M population to the entire species range (Levin, 2000). Conversely, tolerance is likely to have evolved once, at an early stage of the species history, i.e. much earlier than the recent colonization of man-made habitats (Westerbergh, 1994). Two major hypotheses remain equally possible. First, metal tolerance in *A. halleri* could have evolved in response to an early exposure of the species to metal, as has be been suggested for metalloendemics (Kruckeberg & Kruckeberg, 1990). In these species, the adaptation to metal-enriched soils (including metal tolerance acquisition) is supposed to have led to reproductive isolation (Kruckeberg, 1986; Macnair & Gardner, 1998) and metal tolerance is consequently constitutive. Such a hypothesis would be difficult to confirm as *A. halleri* populations have never been recorded as occurring on naturally metal-enriched soils. However, comparative phylogeography including both *A. halleri* and its close relatives (mainly *A. lyrata petrea* and *A. arenosa*), should be able to verify the likelihood of such a scenario. Secondly, Zn tolerance could have evolved in response to the hyperaccumulation trait. Metal hyperaccumulation (accumulation of metal in tissues to very high concentrations) is sometimes assumed to be a defence strategy against herbivores that

Populations	Median value of T_{50} (μM)	NM	NMp	M
(a)				
NM	1171.5	–		
NMp	1390	$W = 180, P = 0.008$	–	
M	1495	$W = 153, P < 10^{-3}$	$W = 59, P = 0.035$	–
ovirensis	1170	$W = 40, P = 0.514$	$W = 8, P = 0.136$	$W = 6, P = 0.023$
	Median value of B			
(b)				
NM	−6.955	–		
NMp	−9.078	$W = 84, P = 0.080$	–	
M	−8.115	$W = 187, P = 0.005$	$W = 48, P = 0.745$	–
ovirensis	−5.887	$W = 19, P = 0.175$	$W = 8, P = 0.136$	$W = 6, P = 0.023$

Table 3 Results of the two-tailed Mann–Whitney tests for comparisons between median values of (a) T_{50} and (b) slope factor B of the different *A. h. halleri* population types (NM, NMp, M) and the *A. h. ovirensis* populations.

NM, nonmetallicolous; NMp, nonmetallicolous in polluted area; M, metallicolous; ovirensis, *A. halleri* subsp. *ovirensis*; *W*, Mann–Whitney statistic.
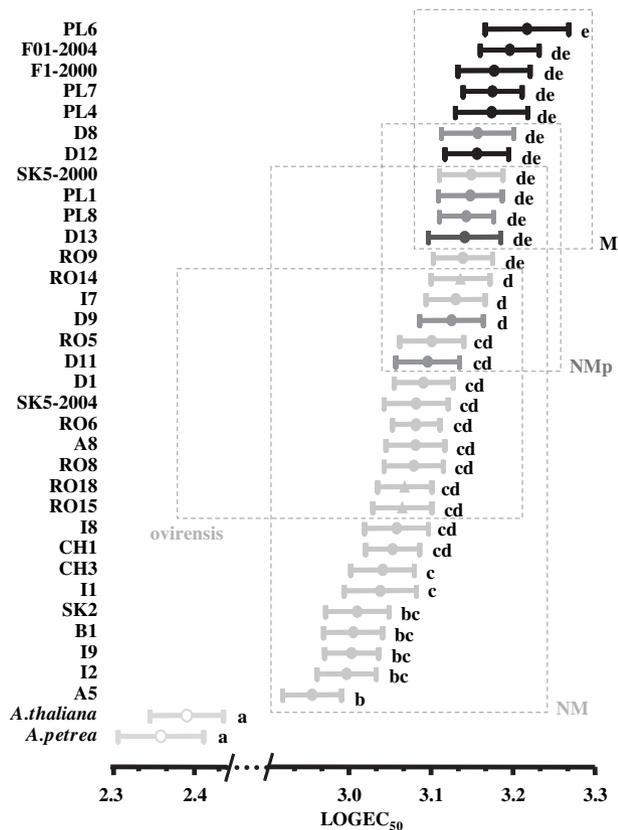
Fig. 5 Comparison intervals for LOGT$_{50}$ given by the GT2-method of multiple comparisons. Different letters indicate a significant difference at the 5% level. Best fit values of control species, *A. h. halleri* and *A. h. ovirensis* are respectively represented by open circles, closed circles and closed triangles. Nonmetallicolous, nonmetallicolous in polluted area and metallicolous populations are respectively indicated in light grey, dark grey and black.

populations. We made it clear that an actual quantitative polymorphism exists between individuals and that a continuum exists in *A. h. halleri* from the least tolerant population to the most tolerant one. Up to now, Zn tolerance genetic architecture in *A. h. halleri* has been studied through analyses of interspecific crosses with *A. lyrata petraea*: one genetic analysis assumed a control by only one major gene and modifiers (Macnair *et al.*, 1999) and a recent QTL analysis detected three genomic regions of similar major effect (W. Willems, C. Godé, D.B. Dräger, M. Courbot, N. Verbruggen, P. Saumitou-Laprade, unpublished data). Therefore, the quantitative polymorphism we observed at the within-species level could be explained by allelic variation in genes present in the identified QTL regions or by additional genes with minor effects not detected in interspecific crosses. Hypostatic modifier genes have commonly been evoked to explain quantitative polymorphism in tolerance in several pseudometallophytes (Macnair, 1993), e.g. copper tolerance in *Mimulus guttatus* (Macnair, 1983; Smith & Macnair, 1998) and *Silene vulgaris* (Schat & Ten Bookum, 1992; Schat *et al.*, 1993; van Hoof *et al.*, 2001).

The observed quantitative polymorphism is a prerequisite for allowing diversifying selection to adapt populations to local conditions (Latta, 1998). Our results strongly attested that there are significant differences in average Zn tolerance among populations. Although no simple correlation could be established between T_{50} measured in controlled conditions and Zn concentration in soils of sites in which seeds were collected (data not shown), heavy metal exposure of populations clearly distinguished groups of populations. M populations were shown to be significantly more tolerant. They were also shown to have a reduced level of within-population polymorphism for Zn tolerance. It has to be noted, however, that the sequential test we used could have generated a bias towards an underestimation of variation in the more tolerant populations (Schat & Ten Bookum, 1992).

The analysis of cpDNA population structure in the northern part of the species range has shown that the populations we sampled in man-made polluted areas have been founded from NM ones located in geographically proximate mountains area (Pauwels *et al.*, 2005). In the absence of strong founder effects (Pauwels *et al.*, 2005), the combination of a higher tolerance level and reduced polymorphism in M populations indicates that natural selection could have acted during the colonization of polluted areas (Meerts & van Isacker, 1997). The location of the most tolerant populations (PL06, F01 and PL04) on the most polluted soils further suggests that the strength of selection was related to the local degree of heavy metal contamination of the soil. Thus, although it is constitutive, Zn tolerance appears to evolve secondarily towards enhanced abilities in M populations. According to Pauwels *et al.* (2005) who concluded that the M population in northern and western Europe had an

could have evolved in the absence of metal exposure (Boyd, 1998). Evolution of hyperaccumulation may have provoked a co-evolution of sequestration capacity which, in return could have improved the tolerance abilities of the hyperaccumulator species. However, the relationships between both Zn and Cd tolerance and hyperaccumulation (Macnair *et al.*, 1999; Bert *et al.*, 2003) and the defensive role of Zn/Cd hyperaccumulation are still highly debated both in *A. halleri* and in *T. caerulescens* (Assunção *et al.*, 2003a; Frérot *et al.*, 2005; Noret *et al.*, 2005). Clearly, the support of this hypothesis will require a better understanding of the genetic make-up of both characters, involving the identification of candidate gene (W. Willems, C. Godé, D.B. Dräger, M. Courbot, N. Verbruggen, P. Saumitou-Laprade, unpublished data).

Evolution of Zn tolerance in Europe

Apart from constitutive tolerance, we revealed significant differences in Zn tolerance abilities of individuals and

independent origin, genetic mechanisms that have been locally selected could differ in separately founded M populations (Schat *et al.*, 1996). Testing of such a hypothesis in *A. halleri* will require QTL mapping for tolerance in recombinant populations from multiple intra-specific crosses between an NM population and independently founded M populations.

Post hoc comparison of $LOGT_{50}$ values demonstrated that many populations could not be significantly distinguished from each other. Comparison made it clear that, although the distinct types of populations differed according to tolerance abilities, they did not actually constitute discrete groups. In particular, an important quantitative polymorphism was observed between and within NM populations, with significant differences between the less and the more tolerant populations, and some NM populations were not distinguished from M ones. The occurrence of quantitative polymorphism among NM populations of *A. h. halleri*, i.e. in the absence of strong metal pressure, is more difficult to interpret in an evolutionary framework. The comparatively high level of tolerance detected in some NM populations (e.g. SK05, RO09, I07 and RO05) suggests that genetic mechanisms conferring enhanced tolerance could be present at relatively high frequency in populations that have not experienced metal exposure in recent times. Overall, the polymorphism observed among NM populations was not related either to the Zn content in soils ($R^2 = 0.002$, $P = 0.867$) or to any available geographic variables. No correlation was found between T_{50} and either latitude ($R^2 = 2.10^{-4}$, $P = 0.953$) or altitude ($R^2 = 0.028$, $P = 0.505$); a weak but significant correlation was found between T_{50} and longitude ($R^2 = 0.241$, $P = 0.038$), although this result strongly suffered from the discontinuous distribution of NM populations according to longitude. Retrospectively, the absence of correlation between T_{50} and altitude in NM populations, which ranges from very low (160 m) to high (1530 m) elevations, attested that altitude could not explain the differences in tolerance abilities between NM and M populations whereas all M populations were at low elevation. Finally, our results also suggest that no selection acts against genetic mechanisms conferring enhanced tolerance in slightly or nonpolluted habitats (Harper *et al.*, 1997b). Obviously, such a hypothesis will require more appropriate experiment (e.g. reciprocal transplantation) to be more rigorously supported.

The features of NMp populations in polluted areas should be understood with respect to the narrow genealogical relationships they share with surrounding M ones (Pauwels *et al.*, 2005). A first assumption is that NMp populations have been founded from M ones and have simply inherited enhanced tolerance. The low metal concentrations in soils and the absence of the directional selection that acts on polluted sites could have secondarily resulted in lower average tolerance levels of NMp population when compared with M ones (Harper *et al.*, 1997a, 1998). Conversely, NMp could have acquired enhanced tolerance from M population through gene flow moving metal tolerance genes. The discussion of both hypotheses will require a population genetic structure analysis at a local scale, ideally replicated in both polluted areas where NMp populations have been mentioned. A combination of both hypotheses is not impossible and would explain the large range of within-population polymorphism observed.

Conclusion

The occurrence of constitutive metal tolerance in a pseudometallophyte species such as *A. halleri* is not in accordance with the classical model accounting for tolerance evolution in pseudometallophytes (Brooks, 1987). In this model, mainly elaborated for the so-called 'mine taxa' whose M populations mostly occur in anthropogenic metal polluted areas and which include most pseudometallophyte species, metal tolerance is expected to have evolved as an adaptive response to recent metal exposure, i.e. in M populations only. In contrast, in *A. halleri*, Zn metal tolerance could result from longer evolutionary history. As a result, the genetics of metal tolerance in these species could strongly differ from mine taxa. Thus, constitutive tolerance could distinguish *A. h. halleri* (as well as *T. caerulescens*, Ingrouille & Smirnoff, 1986; Meerts & van Isacker, 1997) from most pseudometallophytes (Pollard *et al.*, 2002). The status of model species of these species is thus not straightforward and the extrapolation of results obtained from them will have to be done with caution.

Metal tolerance is usually defined as the ability to survive on metaliferrous soils (Antonovics *et al.*, 1971; Macnair & Baker, 1994). Although tolerance tests involving root growth measurement are usually used to infer tolerance abilities of pseudometallophytes (Macnair, 1993), it has to be noted that root growth could only be part of the adaptation to metalliferous soils. Thus, tolerance abilities revealed in NM populations in the present paper do not indicate that all these populations would be able either to lastingly develop on polluted soils or to found M populations. Reciprocal translocations on polluted and nonpolluted sites from a single geographic region using different M and NM accessions characterized in this study are currently in progress in southern Poland. The measurement of life history traits involved in the overall fitness of individuals and including both vegetative growth and reproduction should provide a more integrated view of the capacities of NM and M genotypes to respectively colonize or escape polluted sites.

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