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Research

Zinc and cadmium accumulation in controlled crosses between metallicolous and nonmetallicolous populations of *Thlaspi caerulescens* (Brassicaceae)

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Summary

- Growth and heavy metal (Zn and Cd) hyperaccumulation were investigated in metallicolous and nonmetallicolous Mediterranean populations of *Thlaspi caerulescens* (Brassicaceae), and in offspring from controlled crosses between these populations.
- Seeds for the growth and crossing experiments were collected from a number of sites varying in heavy metal contamination. Tissue Zn and Cd content was determined by atomic absorption spectrophotometry.
- Offspring from crosses between nonmetallicolous populations had the highest Zn concentration (*c.* 30 000 $\mu g\ g^{-1}$), compared with 20 000 $\mu g\ g^{-1}$ for the nonmetallicolous parents. The metallicolous parents and the other crosses had only 10 000 $\mu g\ g^{-1}$. Offspring from crosses including a nonmetallicolous parent still had a significantly higher Zn uptake than the metallicolous parents. A trend towards a higher Cd uptake was observed in offspring from crosses with a metallicolous parent.
- We suggest that the most probable hypothesis is that the differences in Zn hyperaccumulation between crosses could be explained by a monogenic system with two alleles. The dominant allele would restrict Zn hyperaccumulation at 10 000 $\mu g\ g^{-1}$ whereas the recessive allele would be responsible for a two to three-fold increase in Zn hyperaccumulation. Alternatively, the existence of modifier genes could explain the differences between offspring from crosses between nonmetallicolous populations and their respective field parents. The results suggest that plant breeding applied to this species could help to improve Zn phytoextraction.

Key words: *Thlaspi caerulescens*, controlled crosses, zinc accumulation, cadmium accumulation, hyperaccumulation, genetic determinism, phytoextraction.

Introduction

Phytoextraction is a remediation technique employed to remove heavy metals from contaminated soils, using plants called hyperaccumulators (Chaney *et al.*, 1997). These plants are able to tolerate high metal concentrations in soils and to accumulate much more metal in their shoots than in their roots, without suffering from metal toxicity either at high or low concentrations in the soil (Baker, 1981). By successive harvests of the aerial parts of the hyperaccumulator species, the heavy metals concentration in the soil can be reduced. Subsequent incineration of the plant material produces ashes from which metal could be extracted and reused: this is phytomining (Anderson *et al.*, 1999; Leblanc *et al.*, 1999). The concept of a hyperaccumulation threshold was first defined by Brooks *et al.* (1977), based on nickel concentrations generally found in nonaccumulator species, and then applied to other heavy metals. The thresholds currently admitted are 10 000 $\mu g\ g^{-1}$ for zinc, 1000 $\mu g\ g^{-1}$ for lead and 100 $\mu g\ g^{-1}$ for cadmium (Baker & Brooks, 1989; Baker *et al.*, 1994).

One of the most promising plant species for phytoextraction in temperate regions, is *Thlaspi caerulescens* J. & C. Presl. (Brassicaceae) which is able to accumulate very high concentrations of zinc in its aerial parts (Reeves & Brooks, 1983). Recent studies have focused on several aspects of hyperaccumulation in *T. caerulescens*: intra and interpopulation variations (Meerts & Van Isacker, 1997; Escarré *et al*., 2000; Lombi *et al*., 2000), physiology (Krämer *et al*., 2000; Lasat *et al*., 2000, 2001; Pence *et al*., 2000), defense against herbivory (Jhee *et al*., 1999), the genetic basis (Pollard & Baker, 1996), and perspectives for efficient phytoremediation (Robinson *et al*., 1998; Luo *et al*., 2000). Previous works showed that *T. caerulescens*, growing in soil contaminated by zinc and cadmium, can hyperaccumulate up to 10 000 $\mu g\ g^{-1}$ of zinc and up to 1000 $\mu g\ g^{-1}$ of cadmium (Knight *et al*., 1997; McGrath *et al*., 1997; Meerts & Van Isacker, 1997; Escarré *et al*., 2000). Such metal accumulation depends on the growing medium: some individuals growing in hydroponic nutrient solution with added zinc and cadmium, can accumulate up to three times more than in a contaminated soil (Brown *et al*., 1995; Pollard & Baker, 1996; Shen *et al*., 1997). This suggests that it is possible to increase the capacities of hyperaccumulation of *T. caerulescens*. Recently, Meerts & Van Isacker (1997) and Escarré *et al*. (2000) showed that nonmetallicolous populations of *T. caerulescens* tolerated high concentrations of heavy metals and hyperaccumulated up to three times more zinc than populations from contaminated sites. Nevertheless, the above-ground biomass of these individuals was half of the biomass of the metallicolous populations. The ideal phytoextractor would be a plant that is not only able to accumulate the same high zinc concentrations as nonmetallicolous populations, but also produce the same high biomass in toxic environments as metallicolous populations. This ideal combination of traits could be produced by controlled crosses between plants of the two types of origin, establishing the bases for the genetic improvement of the phytoextraction potential in *T. caerulescens*. Such crosses would also provide key information on the genetic basis of the strong difference in zinc hyperaccumulation ability between metallicolous and nonmetallicolous populations.

Until now, most of the genetic studies on species with metallicolous populations have dealt with the genetic determinism of heavy metal tolerance. The model of a system regulated by one or a few major genes associated with genes modifying the dominance, has been formulated for metal tolerance by several authors (Urquhart, 1971; Macnair, 1983; Schat & Ten Bookum, 1992; Macnair, 1993). For example, Schat & Ten Bookum (1992) showed that, in *Silene vulgaris*, the genetic determinism of copper tolerance is monogenic, but the degree of tolerance may be under the influence of two modifier genes. By contrast with heavy metal tolerance, data on the genetic determinism of hyperaccumulation are scarce. As far as we know, only Pollard & Baker (1996) who worked with two metallicolous populations of *T. caerulescens* in Great Britain, showed a continuous variation in zinc hyperaccumulation ability. They suggested that the genetic determinism of hyperaccumulation was polygenic (Pollard, 2000). However, this hypothesis does not explain completely the clear cut difference in hyperaccumulation ability between metallicolous and nonmetallicolous populations, found by Meerts & Van Isacker (1997) and Escarré *et al*. (2000).

In this paper, we present results on the growth and zinc and cadmium hyperaccumulation of offspring issued from controlled crosses including individuals from metallicolous (hereafter M) and nonmetallicolous (hereafter NM) populations, and of their parents, cultivated on a highly contaminated soil from an ancient mining area in the region of Saint Laurent le Minier (Languedoc, France). From these data the following questions were addressed: what kind of genetic determinism, explaining the difference in zinc hyperaccumulation ability between Mediterranean metallicolous and nonmetallicolous populations, can we infer from the controlled crosses we made?; and can classical plant breeding processes improve phytoextraction ability by means of crosses between different populations?

Materials and Methods

Species and study sites

Thlaspi caerulescens J. & C. Presl. is an annual, biennial or pauci-perennial herbaceous member of the Brassicaceae which shows a wide range of morphological variations, that has led to the description of several infraspecific taxa of uncertain taxonomic value (Stace, 1997) which were not confirmed by studies on isozyme variation (Koch *et al*., 1998). The species occurs in Europe from Scandinavia to the Pyrénées and the Carpathians, and from central Europe (Poland) to the British Isles (Tutin *et al*., 1993). The taxon is found at altitudes from a few hundred meters to 2000 m on various substrates including acid or calcareous 'normal' soils (NM populations) and on natural or contaminated sites with high concentrations of heavy metals (M populations).

Seeds of M populations were collected from abandoned mining areas in the Saint Laurent le Minier region (South of France). Three of these metallicolous sites (Malines (MAL), Pommiers (POM), and Saint Bresson (SB)) were abandoned one or two decades ago (Rolley, 2002). The other sites were abandoned much earlier, that is, 100 years ago for Les Avinières (AV) (Demange, 1973) and 700 years ago for 'Moyen Age' (MG) (Bailly Maitre, 1990). The nonmetallicolous individuals (NM populations) were collected in several sites in southern France (Escarré *et al*., 2000): Charnier (CH), Les Infruts (INF), Navacelles (NAV), Saint Michel (SM) and the Séranne Mountain (SER).

Crosses

For the between-populations controlled crosses, plants from two metallicolous and two nonmetallicolous populations

Table 1 Number of controlled crosses between metallicolous and nonmetallicolous populations of *Thlaspi caerulescens* from the Saint Laurent le Minier region (South of France)

		Mother			
		Metallicolous		NonMetallicolous	
		Malines	Moyen Age	Infruts	Séranne
Father					
Metallicolous	Avinières		1	1	1
	Malines	1	1	1	3
	Moyen Age		1	1	
	Pommiers	1	3	1	1
	St Bresson	2		2	1
NonMetallicolous	Charnier	1			1
	Infruts	2			
	Navacelles	3	2	4	4
	Séranne	2	1	1	
	St Michel		1		1

were used as female parents and pollinated with pollen donors from several populations. Table 1 shows the successful crosses in which the maternal origin is named first. We obtained offspring for the four possible cross types: M × M, M × NM, NM × M and NM × NM crosses. Pollinations were carried out by rubbing an open anther on the stigma of the female flower before the opening of its anthers to avoid contamination with self-pollen. Half of the seeds (F1 generation) obtained from the crosses were sown in Petri dishes on moistened filter paper. After germination, 46 seedlings were planted on a substrate made of 50% commercial compost and 50% contaminated soil from the metallicolous site of Saint Laurent le Minier. The substrate had the following heavy metal concentrations (expressed in µg g^{-1} ammonium acetate – EDTA extractable element): 20 700 zinc, 9500 lead and 120 cadmium (Escarré *et al.*, 2000). The plants were grown for 3 months in a heated glasshouse with natural light and watered regularly with distilled water.

The parents used for the F1 crosses were not analysed for zinc and cadmium concentrations, because this requires a significant quantity of tissue, which would have killed these plants or decreased the production of flowers available for the crosses. These plants died at the end of the fruiting period because the species is monocarpic in the region. For this reason, to compare the results of the F1 crosses with those of the parents populations, we used individuals resulting from germination of 18 seeds sampled in three natural metallicolous (MAL, MG and POM) and 20 seeds sampled in three natural nonmetallicolous sites (CH, INF and SM) and collected at the same time as the seeds used as actual parents for the crosses. These individuals were grown under the same conditions as the offspring from controlled crosses. We analyzed only three natural populations by edaphic origin because in a previous experiments (J. Escarré, unpublished; H. Frérot unpublished), there were no differences in zinc accumulation both within metallicolous populations and within nonmetallicolous populations when cultivated in the same substratum (50% compost and 50% contaminated soil).

After 3 months, the green aerial biomass of all plants (parents and offspring) cultivated on the contaminated soil was harvested and dried at 60°C for 3 d. Dried samples were mineralised in a mixture of nitric and perchloric acid and their zinc and cadmium contents determined by flame Atomic Absorption Spectrophotometry. Zinc and cadmium uptake was calculated as the product of metal concentration, times the above-ground biomass.

Statistical analysis

ANOVA were performed to detect differences in biomass, metal hyperaccumulation and metal uptake between crosses and parents, using the GLM procedure of SAS (SAS, 1999). Because some crosses failed, the data set was unbalanced. We thus used type III sums of squares. We pooled together the crosses according to the origin of the father and of the mother because each individual cross produced only a few seeds. The data for parents were pooled by edaphic origin (metallicolous or nonmetallicolous) because previous experiments did not show significant differences within origins with the substrate utilized (J. Escarré unpublished). The differences among means were tested by a Tukey test (SAS, 1999).

Results

Biomass

There were no significant differences in the biomass of offspring from NM × NM crosses and NM parents (1.54 g ± 0.23 and 1.53 g ± 0.15, respectively). The same results were obtained for biomass of offspring from M × M crosses and the M parents (2.65 g ± 0.29 and 2.51 g ± 0.21, respectively) (Fig. 1a). The biomass of the offspring from M × NM crosses was significantly higher than the biomass of the offspring from M × M crosses. Offspring from NM × M crosses had a significantly higher biomass than the offspring from NM × NM crosses, almost twice as much.

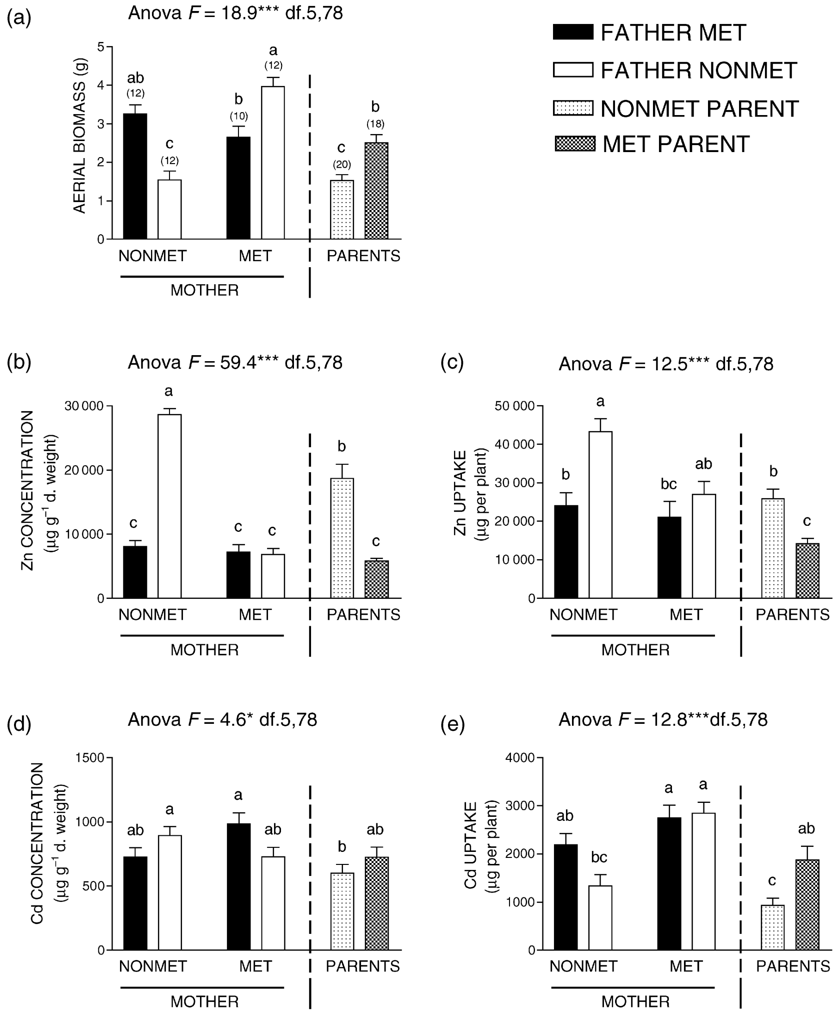

Fig. 1 Mean (± SE) aerial biomass (a), zinc concentration (b), zinc uptake (c), cadmium concentration (d) and cadmium uptake (e) of aerial parts of *Thlaspi caerulescens*. NONMET: individuals from nonmetallicolous populations. MET: individuals from metallicolous populations. PARENTS: individuals from seeds collected in the field. The dashed line separates the results obtained for hybrid plants (left part) from the data for seeds collected in the field (right part). ANOVA: results of one way analysis of variance. Numbers in brackets are the sample sizes. Histograms with the same letter are not significantly different (Tukey test).

Zinc hyperaccumulation and uptake

The zinc concentration in aerial parts of the plants showed very contrasted patterns. Offspring with at least one M parent had significantly lower zinc concentrations (Fig. 1b) than the offspring from NM × NM crosses. The latter had a zinc concentration up to three times higher than the offspring from other crosses and significantly higher than the NM and M parents. For zinc uptake (Fig. 1c), the offspring from NM × NM crosses again showed the highest mean value, but the difference from the offspring from M × NM crosses was not significant.

Cadmium hyperaccumulation and uptake

The mean cadmium concentration (Fig. 1d) was not significantly different among the four types of crosses. Nevertheless, the mean cadmium concentration of the offspring from M × M and NM × NM crosses was significantly greater than the mean of the individuals from the NM parents. Offspring with at least one M parent had the highest mean values of cadmium uptake (Fig. 1e). However they were not significantly different from those obtained with the M parents.

Discussion

Zinc and cadmium uptake and phytoextraction potential

Our results showed that in *T. caerulescens*, the individuals from NM × NM crosses had the lowest biomass among all the offspring but also had a zinc uptake significantly higher than the M or NM parents. In general, when at least one of the parents was from a nonmetallicolous site the zinc uptake of the offspring was higher than that of the metallicolous parents. NM populations could therefore be used in classical procedures for plant breeding in agronomy to increase the zinc extraction abilities of this species. For an efficient zinc phytoextraction, plants have to be sufficiently metal tolerant.

So, NM populations could be crossed with M or NM populations depending on the heavy metal tolerance of offsprings.

Conversely, for cadmium, the differences in concentration of the aerial tissues among offspring from crosses were mostly nonsignificant. Therefore, the differences in cadmium uptake among offspring from crosses were only due to biomass production. If at least one of the parents was metallicolous, the cadmium uptake of the offspring was higher than that produced by the nonmetallicolous parents. However, the possibility of improving cadmium phytoextraction of the populations studied by plant breeding is less clear than for zinc.

The biomass of the offspring was similar to that obtained in a field experiment with metallicolous plants (Frérot, 2001) grown with an addition of organic compost. We can therefore extrapolate the results on metal uptake obtained in experimental conditions to the field. With a density of 150 plants/m^2 (a realistic density according to our preliminary experimental field trials (Frérot, 2001)) and with a zinc uptake between 20 and 40 mg per plant, between 30 and 65 kg of zinc per hectare could be extracted in one harvest. Robinson *et al.* (1998) estimated the zinc extraction ability of the Malines population of *T. caerulescens* to be about 30 kg and 60 kg of zinc per hectare, respectively, without and with chemical fertilizers. From our data it seems reasonable to assess that some of the experimental crosses we obtained are able to extract about 60 kg of zinc per ha without added chemical fertilizers.

We found that the potential for cadmium extraction was between 2.0 and 4.3 kg of cadmium per ha. These values are below the estimations of 4.2–8.4 kg ha^{-1} found by Robinson *et al.* (1998). However their estimations were based on individuals from the Malines population which attain the highest concentrations of cadmium among the populations studied in the region of Saint Laurent Le Minier (Escarré *et al.*, 2000), while our estimations were established from the mean of several populations. Most of these populations grow naturally on soils with lower cadmium concentrations than Malines.

Genetics and evolutionary significance of the difference in hyperaccumulation ability between M and NM populations

The M or NM parents did not show statistically significant differences in Cd hyperaccumulation. The individuals of the F_1 generation showed the same pattern, with very similar values of hyperaccumulation. This does not allow us to make any hypothesis on the genetic determinism of the cadmium hyperaccumulation in *T. caerulescens.*

As far as zinc hyperaccumulation ability is concerned, our results imply that the difference between M and NM populations is heritable, and the data obtained for the F_1 crosses suggest a genetic hypothesis for zinc hyperaccumulation ability in *T. caerulescens.* The offspring from M × M, M × NM and NM × M crosses showed the same hyperaccumulation threshold as the sample of the three populations of M parents. Therefore the restriction of zinc hyperaccumulation by M parents behaves as a dominant trait whereas the increase of zinc hyperaccumulation by NM parents behaves as a recessive trait. We suggest that the hypothesis of a monogenic system with two alleles could thus be responsible for the difference in zinc hyperaccumulation ability between M and NM populations. The hh genotype (NM parents) would determine an increased hyperaccumulation ability (from 20 000 up to 30 000 µg g^{-1}) and the HH and Hh genotypes would restrict zinc hyperaccumulation to about 10 000 µg g^{-1}. Nonmetallicolous individuals would thus be homozygous hh, and the metallicolous individuals would be HH or Hh. However, as no [h] phenotype appeared in the offspring from crosses between different origins, we can formulate the hypothesis that only the HH genotype was present among the metallicolous individuals sampled in the field and used as parents.

Nevertheless, we cannot exclude that several major genes could be involved in the difference between metallicolous and nonmetallicolous populations. In the case of the NM populations, all the individuals would be homozygous for recessive alleles at all loci. For the metallicolous populations, if the restriction of hyperaccumulation involved many genes exhibiting one or two dominant alleles, then levels of hyperaccumulation between the crosses with either one or two metallicolous parents (i.e. M × M, M × NM, NM × M) would be significantly different. But this was not the case.

However, a hypothesis of monogenic determinism does not explain the difference in zinc hyperaccumulation between the NM × NM crosses and their respective parents. This difference was lower than that between NM × NM crosses and the other crosses or the M parents. We suggest that this difference could be due to modifier genes favouring zinc hyperaccumulation levels above 10 000 µg g^{-1}.

In conclusion, if the genetic determinism of a zinc hyperaccumulation level greater than 10 000 µg g^{-1} actually appears monogenic, then it will be possible to search a molecular marker linked to this character. Also, this suggests that genetic improvement of phytoextaction could be rapid owing its simple genetic basis. Finally a high number of individuals from a large array of ecogeographically distinct populations could be screened in order to estimate the frequency of the recessive h allele which confers this character, and experimentally test the trade-offs associated with hyperaccumulation, both on polluted and normal soils, and their adaptive signification.

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