Influence of zinc hyperaccumulation on glucosinolates in *Thlaspi caerulescens*

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**Summary**

* Previous investigations suggest that in species of the Brassicaceae hyperaccumulation of heavy metals might provide an ecological advantage by protecting the plants against herbivores and/or pathogens while lowering the glucosinolate content. Few analytical data on glucosinolate concentrations in hyperaccumulators are available for supporting this ‘trade-off’ hypothesis.

* This is the first report on the influence of zinc (Zn) hyperaccumulation on the concentrations of individual glucosinolates in *Thlaspi caerulescens* exposed to different Zn concentrations.

* The most abundant glucosinolate within both roots and shoots was p-hydroxybenzyl glucosinolate (sinalbin). Zn hyperaccumulation decreased sinalbin concentrations in shoots, whereas root concentrations increased with Zn accumulation. These changes in sinalbin concentrations were mainly responsible for Zn-induced alterations of total glucosinolate contents. Quantitatively less important was a Zn-induced decrease of indolylglucosinolates observed in both roots and shoots and that of 3-butenylglucosinolate found in roots.

* The results presented here support the view of a trade-off between Zn and glucosinolates in shoots but not in roots of *Thlaspi caerulescens*.

**Key words:** Hyperaccumulation, glucosinolate, sinalbin, *Thlaspi caerulescens*, zinc.


**Introduction**

The phenomenon of metal hyperaccumulation in plants has attracted particular interest in the last years, because plants that combine high metal tolerance with an extraordinary ability for metal uptake may be useful for phytoextraction technologies (Baker et al., 1994; Raskin et al., 1994). However, the ecological significance of hyperaccumulation is still under investigation. Several recent studies relate metal hyperaccumulation to increased defence against herbivores and fungal or bacterial infections (Boyd & Martens, 1994; Boyd et al., 1994; Pollard & Baker, 1997; Davis & Boyd, 2000; Ghaderian et al., 2000). The exceptionally high leaf metal concentrations seem to act as either feeding deterrents or toxicants thus providing advantage over nonhyperaccumulator species on metalliferous soils (Boyd & Martens, 1994; Pollard & Baker, 1997).

Many hyperaccumulator species (e.g. those of the genera *Alyssum* and *Thlaspi*) are from the Brassicaceae family. Glucosinolate production is a common feature of plants from this botanical group. High glucosinolate levels in Brassicaceae species that hyperaccumulate Ni (Sasse, 1976) or Zn (Mathys, 1977) have been described.

It has been suggested that glucosinolates may play a role in Zn tolerance mechanisms (Mathys, 1977) or that the pool of these sulphur containing compounds may serve as a source for Ni-binding substances (Sasse, 1976) in *Alyssum bertolonii*. As pointed out by Ernst (1990), however, observations that high Ni concentrations lowered glucosinolate concentrations in *Alyssum* and that high Zn levels reduce sulphotransferase activity are convincing arguments against these hypothesis.

Glucosinolates have been related to plant defence mechanisms for many years (Schnug, 1990; Wallsgrove et al., 1999). In metal hyperaccumulator species, however, high metal concentrations in tissues have been suggested to provide protection while organic defences are saved. Feeding preference of slugs and caterpillars for leaves of the Zn hyperaccumulator *Thlaspi caerulescens* with low Zn concentrations over high Zn leaves suggests an antifeedant effect by high metal concentrations (Pollard et al., 1993).
& Baker, 1997). High Ni accumulation also seems to play a role in defence against herbivores in the Ni-hyperaccumulator Streptanthus polygaloides (Davis & Boyd, 2000). Ernst (1990) suggested that high glucosinolate levels in metallophytes of the Brassicaceae may not primarily function against herbivores, being already killed by the high metal concentrations, but as a defence mechanism against pathogenic fungi. In fact, upon tissue damage by fungal infection, glucosinolates are catabolized by myrosinases producing compounds with fungitoxic or fungistatic effects (Matile, 1980; Schnug, 1990; Mayton et al., 1996). Investigations on the pathogenicity of Pythium species in Ni-hyperaccumulator and nonaccumulator species of Alyssum, however, indicate that protection against fungal infection may also be conferred by high Ni levels in the tissues (Ghaderian et al., 2000).

At present, the relative importance of glucosinolates and metal accumulation in defence against herbivores and fungi in hyperaccumulator species is not clear. Moreover, to date the discussions on the role of glucosinolates in defence and tolerance mechanisms in metal hyperaccumulators are based on total glucosinolate content. As different glucosinolates may have different activity in biological interactions (Wallschweiler, 1998), investigations into the influence of metal uptake on glucosinolate patterns in hyperaccumulators may provide new insight into this problem.

To the best of our knowledge, this is the first report on the influence of metal uptake on the levels of individual glucosinolates in a hyperaccumulator species.

Materials and Methods

Plant material and Zn analysis

Plants of Thlaspi caerulescens J. & C. Presl were grown for 3 wk in aerated nutrient solution supplemented with ZnSO4 7H2O to obtain three different Zn treatments: 1.5, 500 and 1000 μM Zn. The experiments were performed in a clean, controlled-environment chamber. Growth conditions were as described by Tolrà et al., 1996. During the experiment and upon harvest, plants were checked for absence of fungal and insect attack.

Zinc concentrations in roots and shoots were analysed in triplicate by ICP-ES following acid digestion of the plant material (Tolrà et al., 1996). Upon harvest the plant material for glucosinolate determinations was weighed and immediately frozen in liquid N2. After freeze drying the material was stored at -80°C.

Total glucosinolate contents

Total glucosinolates were extracted with 70% (v/v) aqueous methanol in a boiling water bath. After centrifugation the pellet was re-extracted. The joint liquid phases were treated with myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1.) (Sigma, St. Louis, MO, USA). The released D-glucose was quantified using an enzyme-based analytical kit (Boehringer, Mannheim, Germany) (Tolrà, 1997). The effectiveness of the extraction method was validated using certified reference rapeseeds (CRM 367) from the European Community Bureau of Reference (BCR, Brussels, Belgium) as standard material. The certified glucosinolate content of this material is 102 ± 3 μmol g−1. Our analytical procedure yielded a mean ± standard deviation value of 106 ± 8 μmol g−1 (n = 3).

Extraction and separation of individual glucosinolates

The analysis of individual glucosinolates was performed as previously described (Tolrà et al., 2000). In brief, glucosinolates were extracted from freeze dried plant material with 70% (v/v) aqueous methanol in a boiling water bath for 5 min. After cooling and centrifugation, the proteins of the supernatant were precipitated with lead and barium acetate. For the enzymatic desulphatation the extract solution was loaded onto a DEAE-Sephadex A-25 Column and glucosinolates were treated in the column with aryl sulphatase (H-1 type from Sigma, St. Louis, MO, USA) following the method of Minchinton et al. (1982). The eluate was freeze-dried and stored at −80°C. For HPLC analysis samples were dissolved in water and passed through a 0.45-μm filter.

A liquid chromatograph equipped with a diode array detector Model HP 1090 (Hewlett Packard, CA, USA) was used. For the analysis 25 μl samples were injected onto a Lichospher 60 RP Select B column (Merck, Darmstadt, Germany). The mobile phases used for the elution samples were acetonitrile and water containing 0.5% (v/v) acetic acid. The gradient elution was performed as follows: from 100% water and 0% LC-grade acetonitrile to 88% (v/v) water and 12% (v/v) LC-grade acetonitrile in 65 min, at flow rate of 1 ml min−1.

Mass spectrometric analysis

LC-APCI-MS in the positive mode was used for the determination of glucosinolates. A HP 1100 mass spectrometer (Hewlett Packard, CA, USA) equipped with an APCI interface and APCI spray chamber was used. Optimization of the instrument parameters for glucosinolates detection was performed using commercial sinigrin (Sigma-Aldrich, St. Louis, MO, USA) (Tolrà et al., 2000).

Statistics

The experiment was performed three times, yielding similar results. Results reported are from one representative experiment. In each experiment Zn concentrations were applied to at least 12 plants per treatment. Concentrations of total glucosinolates and of Zn in roots and shoots were each determined on three different individuals. Quantification of individual glucosinolates was performed in duplicate in two individual plants per treatment. Results are given as mean ± SD. Statistical treatment

of data was performed using the PC program STATISTICA (Statsoft, Inc., USA). Data were treated by ANOVA; the significance of differences between means were determined by Tukey HSD test \( (P < 0.05) \).

**Results**

Growth response of *Thlaspi caerulescens* to the high Zn supply was as previously described (Tolrà *et al.*, 1996). Exposure to 500 µM Zn enhanced the performance; no toxicity symptoms were observed in plants treated with 1000 µM Zn. *Thlaspi caerulescens* accumulated high concentrations of Zn within both roots and shoots. While in shoots Zn concentrations increased with the Zn supply, in roots a saturation of Zn concentrations for the highest Zn treatment was observed (Fig. 1).

Exposure to increasing Zn concentrations decreased the concentrations of total glucosinolates (Fig. 2) in shoots. In roots total glucosinolate concentrations increased with increasing Zn tissue concentrations.

Table 1 shows the trivial and semisystematic names of the individual glucosinolates in roots and shoots of *Thlaspi caerulescens* plants. The only glucosinolate found in all treatments within both roots and shoots was p-OH-benzylglucosinolate. Another aromatic glucosinolate, 2-phenylethylglucosinolate, was observed in shoots of control plants only. The only alkenylglucosinolate detected, 3-butenylglucosinolate, was present in roots of plants from all treatments. The methoxylated indole glucosinolates found in roots and shoots of control plants differed in the position of the MeO group (1-MeO in roots and 4-MeO in shoots).

Quantification of individual glucosinolates showed that the p-OH-benzylglucosinolate (sinalbin) was by far the most abundant glucosinolate in all organs and treatments (Fig. 3). Where present, the concentrations of 3-butenyl- (Fig. 4) and 2-phenylethyl-glucosinolates (Fig. 5) were about one order of magnitude lower. Lowest concentrations were found for indolylglucosinolates (Fig. 5).

Different effects of Zn hyperaccumulation on the levels of individual glucosinolates in roots and shoots were observed. While exposure to high Zn concentrations decreased the concentrations of the aromatic glucosinolates in shoots, in roots a significant increase of p-OH-benzylglucosinolate levels was found (Fig. 3).

Concentrations of 3-butenylglucosinolate, the other major root glucosinolate, were hardly affected by the Zn supply (Fig. 4). The concentrations of indolylglucosinolates found in roots and shoots of control plants decreased to undetectable levels in plants exposed to high Zn concentrations (Fig. 5).
Table 1 Trivial and semisystematic names of glucosinolates found in roots and shoots of *Thlaspi caerulescens*. In organs marked with an asterisk the glucosinolate was only present in certain treatments.

<table>
<thead>
<tr>
<th>Semi-systematic name</th>
<th>Trivial name</th>
<th>Abbreviation</th>
<th>Organ</th>
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<tr>
<td>Alkenyl</td>
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<tr>
<td>3-butenyl</td>
<td>Gluconapin</td>
<td>3-B</td>
<td>Root &amp; Shoot*</td>
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<tr>
<td>Aromatic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-hydroxybenzyl</td>
<td>Sinalbin</td>
<td>OH-benz</td>
<td>Root &amp; Shoot</td>
</tr>
<tr>
<td>2-phenylethyl</td>
<td>Gluconasturtin</td>
<td>2-Pe</td>
<td>Shoot*</td>
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<td>Indole</td>
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<tr>
<td>4-methoxy-3-indolymethyl</td>
<td>4-MeO-Glucobrassicin</td>
<td>4-MeO</td>
<td>Shoot*</td>
</tr>
<tr>
<td>1-methoxy-3-indolymethyl</td>
<td>1-MeO-Glucobrassicin</td>
<td>1-MeO</td>
<td>Root*</td>
</tr>
</tbody>
</table>

Discussion

Relatively high total glucosinolate concentrations have been reported in metal hyperaccumulator species of the Brassicaceae family (Ernst, 1990). Early investigations reported higher total glucosinolate concentrations in a Zn-tolerant than in a Zn-sensitive population of *Thlaspi alpestre* from the Vosges (Mathys, 1977). Also in the Ni tolerant serpentinophyte *Alyssum bertolonii* higher glucosinolate concentrations were found than in the Ni-sensitive *Alyssum argenteum* (Sasse, 1976). Results reported here, however, do not support a general relation between the potential for metal hyperaccumulation and constitutively high glucosinolate contents. The total glucosinolate concentrations in the *Thlaspi caerulescens* of this study were in the same order of magnitude as those reported for other Brassicaceae species (Schnug, 1990; Doughty et al., 1991; Wallsgrove et al., 1999) that are not able to hyperaccumulate potentially toxic metal concentrations. A relatively low total glucosinolate concentration (0.4 μmol g⁻¹ f. wt) has also been reported in leaves of the Ni hyperaccumulator *Alyssum pintodasilvae* (Tolrà et al., 1998). Moreover, recent investigations in *Spreptanthus* (Brassicaceae) found that total glucosinolate contents were significantly lower in the Ni-hyperaccumulator *Spreptanthus polygaloides* than in the nonhyperaccumulator *Spreptanthus insignus* (Davis & Boyd, 2000).

Several investigations have shown that the accumulation of high Ni or Zn concentrations can protect plants against attacks by pathogens and herbivores (Boyd & Martens, 1994; Boyd et al., 1994; Pollard & Baker, 1997; Ghaderian et al., 2000). Taking into account the high biosynthetic costs of glucosinolates (Ernst, 1990), a substitution of organic defence based on glucosinolates by inorganic, metal-based, defence may be advantageous (Boyd, 1998). In fact, a substantial decrease in glucosinolate concentrations in metallophytes that had hyperaccumulated Ni or Zn has already been observed by Sasse (1976) and Mathys (1977). By contrast, Ni hyperaccumulation did not influence glucosinolate levels in the serpentinophyte *Spreptanthus polygaloides*, where the trade-off between organic and Ni-based defence has been suggested to be a constitutive rather than substrate–induced (Davis & Boyd, 2000).

This study is the first that reveals that glucosinolate concentrations in roots and shoots may clearly respond in a different way to enhanced Zn accumulation: while decreased glucosinolate levels were observed in leaves of plants that had accumulated extremely high Zn concentrations, glucosinolate levels in roots increased with Zn accumulation (Fig. 3).

At the present stage of knowledge it is not clear to what extent these differences in the response to Zn of root and shoot glucosinolate levels are consequences of Zn-induced changes in sulphur pools or responses related to defence. High...
Zn concentrations have been found to inhibit sulphation of desulphoglucosinolates in cress seedlings (Glendering & Poulton, 1988). In epidermal vacuoles of leaves of *Thlaspi caerulescens* high Zn concentrations were associated, among other storage forms, with compounds containing high sulphur concentrations (Vázquez *et al.*, 1994). However, defence-related differences between root and shoot glucosinolate contents cannot be discarded. From a theoretical point of view, differences in the role of organic and metal-based defences in roots and shoots are to be expected. For surviving in metal-rich soils pathogens must have evolved metal tolerance (Boyd *et al.*, 1994; Ghaderian *et al.*, 2000). Therefore, in roots a metal-based defence may be inefficient against soil-borne diseases. By contrast, in shoots, metal accumulation would be effective as deterrent or poison against herbivores that may have adapted or not to high glucosinolate contents but are sensitive to high metal concentrations.

Identification of individual glucosinolates showed that sinalbin (p-OH-benzylglucosinolate) was the most abundant glucosinolate within both roots and shoots (Fig. 3). Zinc-induced enhancement of the total glucosinolate contents in roots and their decrease in shoots (Fig. 2) can mainly be attributed to changes in the concentrations of this aromatic glucosinolate. Changes in the levels of alkenyl- and indolyl-glucosinolates caused by Zn hyperaccumulation were quantitatively less important. However, differences in the response of individual glucosinolates to high Zn were observed. These differences may reflect Zn-induced changes in either or both the availability of aminoacid precursors (Cakmak *et al.*, 1989; Domingo *et al.*, 1992) for glucosinolate synthesis or the alteration of one of the substrate specific biosynthetic steps (e.g. aldoxime biosynthesis catalysed by cytochrome P-450, flavoproteins, or peroxidases) in the synthesis of glucosinolates with different side chains (Larsen, 1981; Schnug, 1990; Wallsgrove *et al.*, 1999). A specific influence of Zn is suggested by the fact that in roots the levels of the tyrosine-derived p-OH-benzylglucosinolate increased (Fig. 3), while a decrease of both the indolylglucosinolates (Fig. 5) that are synthesized from tryptophane and the methionine-derived alkenylglucosinolate (Fig. 4) was observed.

Different glucosinolates have different biological activity against pathogens and herbivores. Moreover, the content of particular glucosinolates may determine the response to specialist insects (Wallsgrove *et al.*, 1999). Therefore, Zn-induced changes in glucosinolate patterns may have complex consequences for the defence responses in *Thlaspi caerulescens*. Insect or fungal attacks seem to enhance especially levels of aromatic and indole glucosinolates in Brassicaceae, while aliphatic glucosinolates may even be lowered (Dougherty *et al.*, 1991). Zinc hyperaccumulation decreased concentrations of p-OH-benzyl- and of 1-MeO-indolyl-glucosinolates in the leaves of *Thlaspi caerulescens* (Figs 3 and 5). Investigations with three different herbivores showed preferred feeding on leaves of *Thlaspi caerulescens* with low Zn concentrations (Pollard & Baker, 1997). This result, in combination with our data, supports the view of a trade-off between Zn hyperaccumulation and glucosinolates as feeding deterrents in shoots of *Thlaspi caerulescens*.

By contrast, Zn-induced changes in the glucosinolate patterns of roots are not in line with the ‘trade-off’ hypothesis in these underground plant parts. In roots of *Thlaspi caerulescens* Zn hyperaccumulation increased the concentrations of sinalbin (Fig. 3). This effect may contribute to increased defence against pathogens. The hydrolysis product of sinalbin, p-OH-benzilisothiocyanate has been identified as the predominant antifungal compound in *Brassica kaber* (Schreiner & Koide, 1993). Sinalbin also was the predominant glucosinolate induced by inoculation with *Glomus mosseae* in *Sinapis alba* (Vierheilig *et al.*, 2000). To what extent Zn hyperaccumulation and enhanced sinalbin concentrations in roots may have additive or even synergistic effects (Boyd, 1998) in the defence of roots of *Thlaspi caerulescens* against pathogens remains to be established.

**Acknowledgement**

We thank Dr A. J. M. Baker for the gift of *Thlaspi caerulescens* seeds. This work was supported by the Spanish Government (DGICYT, PB 97--0163-C02-01).

**References**


