How does *Thlaspi goesingense* accumulate heavy metals?

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Despite their great future potential, a multitude of questions remain to be addressed before hyperaccumulators can be used to clean up contaminated sites. We used a scanning electron microscope and light microscopy to address the question how *Thlaspi goesingense* accumulates metals. Hyperaccumulation of nickel by *T. goesingense* was confirmed: the recorded concentration was ten times beyond the threshold that defines a nickel hyperaccumulator. The pathogen/herbivore defence hypothesis can indirectly be confirmed because cuticular striations increased when the nickel concentration decreased. The large, elongated epidermis cells in *T. goesingense* indicate that metals are sequestered and immobilized because these cells correlated with elevated nickel concentrations. These cells are less frequently encountered in *T. arvense*, a non-hyperaccumulator. Exceptions were recorded in the leaves of the inflorescence axis: the nickel concentration here was relatively high but only a few elongated cells were present. The high amount of nickel and zinc in the plant confirms the metal tolerance of *T. goesingense*. The disposal-from-the-Plant-Body Theory can also be confirmed because the leaves of the inflorescent axis, which are lost after flowering, accumulated high amounts of nickel. Detoxification is by disposal of nonessential plant organs. Other mechanisms of disposal are unlikely because no trichomes or other adaptations were recorded.

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Trotz zahlreicher Untersuchungen ist bis heute nicht geklärt, ob Akkumulatorpflanzen in der Lage sind, schwermetallkontaminierte Böden nachhaltig zu sanieren. Unter diesen Akkumulatorpflanzen findet sich auch *Thlaspi goesingense,* das mit Hilfe der Lichtmikroskopie und der Rasterelektronenmikroskopie untersucht wurde, um festzustellen, wie die Pflanze Schwermetalle speichert. Im Fokus des Interesses ist das Element Nickel, da *T. goesingense* als Ni-Hyperakkumulator in zahlreichen Studien beschrieben ist. Das konnte auch in vorliegender Arbeit bestätigt werden. Als Depots mit hoher Speicherkapazität für Nickel werden die großen Epidermiszellen der Blätter angesehen. Eine Ausnahme bilden die Blätter der Blütenstandsachse: Diese haben einen relativ hohen Nickelgehalt, aber nur wenige große Epidermiszellen. *T. arvense,* eine nicht schwermetallresistente Pflanze, besitzt dagegen keine großen Epidermiszellen in den Blattorganen. Offenbar werden die Schwermetalle (Nickel und Zink) nach der generativen Phase vermehrt in jene Organe verlagert, die am Ende der Vegetationsperiode abgeworfen werden. Auf diese Art und Weise kann der Schwermetallgehalt in der Pflanze deutlich reduziert werden.

Keywords: *Thlaspi goesingense*, *Thlaspi arvense*, Hyperaccumulator, Nickel, Zinc, Anatomy, SEM.

Introduction

The last two decades have seen an increased interest in hyperaccumulators such as *Thlaspi goesingense* Hálácsy, as they can potentially be used to clear up contaminated sites. As early as in 1983 it was established that *Thlaspi goesingense* is a hyperaccumulator. With concentrations of up to 2680 mg kg^{-1} (0.27 %) in roots and 12,400 mg kg^{-1} (1.2 %) in leaves, both fresh *T. goesingense* and herbarium material has been shown to hyperaccumulate nickel (REEVES & BROOKS 1983, REEVES & BAKER 1984, PERSANS et al. 2001, AIGNER 2005). Nickel is not the only metal accumulated by *T. goesingense*: zinc is accumulated in high concentrations, and the accretion of cadmium is still under study. This paper investigates how *T. goesingense*, as a known hyperaccumulator, accumulates high quantities of heavy metals. Several explanations have been forwarded as to why plants

hyperaccumulate these toxic metals. These are listed in table 1 with the respective references (BOYD and MARTENS 1998 a).

The Pathogen/Herbivore Defence Hypothesis suggests that accumulated metals deter herbivores and/or pathogens. Hyperaccumulation is in this case a defensive strategy. One of latest and most interesting studies in this field established that an absence of nickel increased the susceptibility of *T. goesingense* to the mildew *Erysiphe cruciferarum* (a powdery mildew) (FREEMAN et al. 2005).

The inadvertent uptake hypothesis suggests that the uptake of metals is a by-product of other physiological processes and has no immediate use for the plant itself. The interference hypothesis, on the other hand, describes the phenomenon of competition avoidance by hyperaccumulation. Interference is reminiscent of allelopathy. The only difference is that hyperaccumulators release metals. Plants can, however, also lose (disposal/excretion theory) the metals they have accumulated (ERNST 1972). JAFFRÉ (1980) reported that in *Geissois pruinosa, Hybanthus austrocaledonicus* and *Psychotria douarrei*, 90% of the accumulated metal concentrations were lost over the course on one year. The exact mechanisms behind this loss were not discovered. Certain plants also excrete metals from their

Tab. 1: Overview of the different hyperaccumulation theories, modified after BOYD & MARTENS (1998a, cited lit. see there). – Übersicht verschiedener Schwermetall-Toleranz-Hypothesen, nach BOYD & MARTENS (1998a, zit. Lit. s.dort), verändert.

Hypothesis	Papers
Inadvertent uptake	SEVERNE & BROOKS 1972 BAKER & WALKER 1989
Disposal from plant body/ Excretion	ERNST 1972 WILD 1978 JAFFRÉ 1980 BAKER 1981 FARAGO & COLE 1988 PSARAS et al. 2000
Drought resistance	SEVERNE 1974 BAKER & WALKER 1990 ROBERTSON 1992 MESJASZ-PRZYBYLOWICZ et al. 1996 BOYD & MARTENS 1998(b)
Interference	BAKER & BROOKS 1989 GABRIELLI et al. 1991 WILSON & AGNEW 1992 GABRIELLI et al. 1997
Metal tolerance	ANTONOVICS et al. 1971 BAKER 1981, 1987 KRUCKEBERG et al. 1993 EBBS et al. 2002 KRÄMER et al. 1997
Enhanced ability to transport metals	KRÄMER et al. 1997 HIMMELBAUER et al. 2005
Pathogen/herbivore defence	REEVES et al. 1981 ERNST 1987 ERNST et al. 1990 FREEMAN et al. 2005 b

guard cells and/or trichomes (PSARAS et al. 2000) or from non-essential plant parts that are shed (PSARAS & MANETAS 2001). Another possibility is that hyperaccumulators have the enhanced ability to transport metals from the root to the shoot (KRÄMER et al. 1997). The drought resistance hypothesis focuses on the ability of hyperaccumulators to tolerate drought periods by reducing cuticular transpiration (BOYD AND MARTENS 1998 b). Finally, metal tolerance has been proposed: here, metals are sequestered and immobilised (KRÄMER et al. 1997, EBBS et al. 2002 and ASEMANEH et al. 2006). This study examines which of these hypotheses is viable for the accumulation in the hyperaccumulator *T. goesingense*.

Material and Methods

Specimens of *Thlaspi goesingense* (Hálácsy, Brassicaceae) were collected from Redlschlag, a natural serpentine site in Burgenland, Austria. The collected specimens were in their second vegetation period and of the same age. The plants were held at an outdoor location at the Vienna Biocentre until 16 October 2006. The plants were then successively relocated to the greenhouse. Flowering specimens of *T. arvense* from Poland were randomly sampled in the herbarium of the University of Leiden (The Netherlands). Flowering *T. goesingense* specimens for the anatomical study were first thoroughly rinsed with water and stored in 50% alcohol for one week. Leaf samples were taken from the middle of the lamina. Sections of mature leaves were made (thickness 40 µm) using a Reichert sledge microtome. Sections were stained with safranine/astrablue and mounted in Canada balsam. *T. arvense* specimens were rehydrated, by boiling, and stored in 50% alcohol. These sections were first bleached, then stained using Etzoldscolour solution and mounted in glycerine-gelatine. Photographs were recorded with an SIS Colorview 1 digital camera mounted on a Leica BX51. Measurements were performed with AnalySIS docu software.

The thickness of the young leaves of *T. goesingense*, and their upper (adaxial) and lower (abaxial) epidermis, was measured at 20 randomly selected spots. For mature leaves, 23 areas were randomly selected. Each area was measured between 8 and 14 times. This depended on the visually determined variation in thickness. For leaves of the inflorescence axis, 8 spots were selected. For *T. arvense* leaves, 10 spots were selected. The elongated epidermis cells were counted over a standard length of 714.14 µm: this was the distance of the lamina visible at a 40-times magnification in the AnalySIS docu software.

A scanning electron microscope (SEM) was used to study the metal concentrations (on a dry weight basis) and their distribution in the leaves. Randomly selected plants were harvested on the following dates: 17 and 24 May, 5 July, 17 August, 2 October and 28 November 2006. See table 2 for the organs that were collected at each specific date.

Samples were rinsed using water and deionised water. The specimens of 17 May and 28 November were air-dried in an desiccator; all other specimens were freeze-dried. Young and mature rosette leaves were always selected and, if possible, leaves of the inflorescence axes, petals and inflorescence axes at different heights were also sampled. Subsequently, 4–10 cross-sections were made using a razorblade. For leaf samples, the middle of the lamina was selected. This method has been successfully used in an earlier study (KÜPPER et al. 2001). These samples were then mounted on aluminium stubs which were covered with carbon tabs and coated with carbon using a Paar EPA 101 B50/200R. The distribution analysis was preformed at least 15 times per plant part with the EDXA program eDXi in a Philips XL 20 SEM. For the tissue distribution, at least 7 sites in the

Tab. 2: Average nickel concentration in dry weight percentage in *T. goesingense* N.R. = Not recorded; N.A. = Not Available. Letters indicate significance at $P < 0.05$ (different letters indicate significance). – Durchschnittliche Nickel-Konzentration (% TG) in *T. goesingense* N.R. = Keine Angaben N.A. = Organ nicht verfügbar. Buchstaben zeigen Signifikanzen.

Harvest (2006)	Mature leaves	Young leaves	Leaves of the inflorescence axis	Lower inflore-scence axis	Middle inflore-scence axis	Higher inflore-scence axis	Petals
17 May	0.94 ± 0.05 a	N.R.	0.74 ± 0.03 a b	N.A.	N.R.	N.A.	0.45 ± 0.03 b
24 May	2.14 ± 0.08 a	0.79 ± 0.03 b	2.36 ± 0.14 a	N.R.	0.09 ± 0.02 c	0.26 ± 0.02 c	N.A.
4 July	0.25 ± 0.02 a	0.38 ± 0.03 a	N.R.	N.R.	N.A.	N.A.	N.A.
17 August	0.11 ± 0.01 a	0.04 ± 0.01 a	N.A.	N.A.	N.A.	N.A.	N.A.
2 October	0.23 ± 0.01 a	0.06 ± 0.01 a	N.A.	N.A.	N.A.	N.A.	N.A.
28 November	0.47 ± 0.02 a	0.21 ± 0.01 a	N.A.	N.R.	N.A.	N.A.	N.A.

mesophyll and epidermis were randomly selected. In the vascular bundles, fewer spots were available. For all measurements, the voltage was set at 25 kV and a working distance of 12 mm was selected. Photographs and measurements performed on SEM material were analysed with the program Scandium.

Results

Anatomy

The leaves of *Thlaspi goesingense* are bifacial and dorsiventral. The palisade parenchyma is constructed of multiple layers and the cells are small and almost round. The mesophyll varies from compact in rosette leaves to lacunar in the leaves of the inflorescence axis, where the spongy tissue is also compact but the palisade tissue is very lacunar. In *T. arvense* the leaves are also bifacial, dorsiventral and the mesophyll is compact. In both species the vascular bundles are delimited by a bundle sheath of parenchyma cells.

All examined *T. goesingense* specimens possess large, elongated cells in their epidermis (figure 1). These cells are present in the leaves of the inflorescence axis, young rosette and the mature rosette leaves. The examined *T. arvense* specimens, however, principally lack these cells, except in the region of the vascular bundles. Examination of *T. goesingense* clearly reveals that these cells are concentrated around the vascular bundles. The number of elongated cells in the abaxial and adaxial epidermis of the mature leaf of *T. goesingense* is not significantly different ($P>0.50$). An average value of 2.35 elongated cells per unit (unit length: 714 µm) is recorded in the adaxial epidermis compared to 2.31 for the abaxial side. The same trend is evident in the young leaves; there is also no significant difference ($P<0.20$) between the average number of elongated cells in the abaxial epidermis (0.50 cells per unit) and in the adaxial epidermis (0.75 cells). The different number of elongated cells in the young (1.25 cells) versus mature leaves (4.48 cells per unit) is, however, highly significant ($P<0.0001$). The leaves of the inflorescence axis contained only a few elongated epidermis cells, so that no difference between the abaxial and adaxial side was documented. Highly significant ($P<0.001$), however, is the difference in the epidermis thickness of the young and mature leaves (corrected for size). The

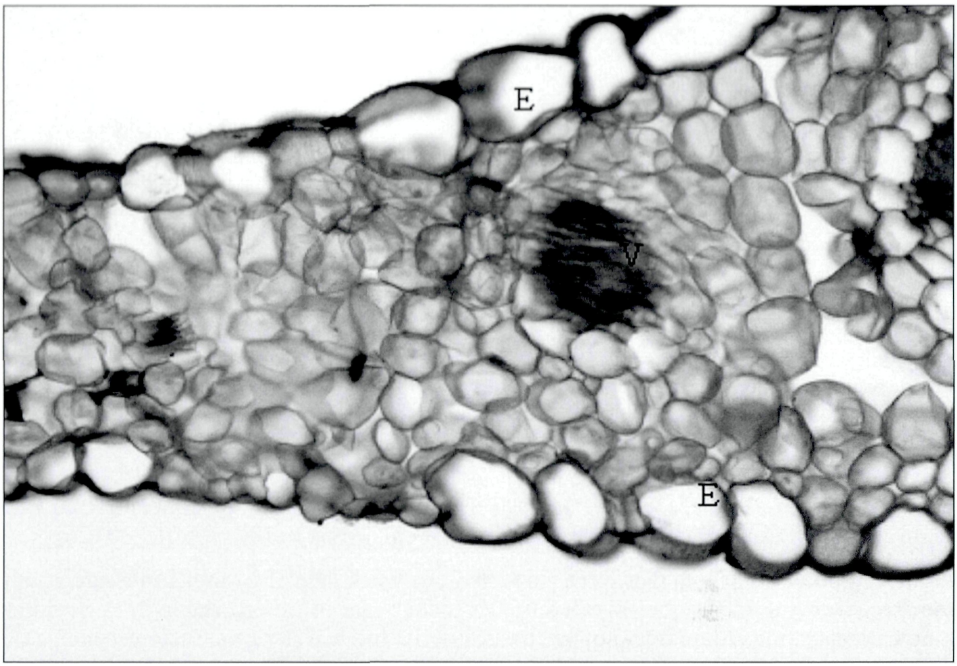

Fig. 1: Large, elongated epidermis cells (E) in the cross section of a mature leaf of *Thlaspi goesingense*. Magnification 60x, E, Epidermis V, vascular bundle. – Große Epidermiszellen (E) im Blattquerschnitt von *Thlaspi goesingense*. Vergrößerung 60 x E, Epidermis V, Gefäßbündel.

overall thickness of the lower and upper epidermis in percentage of total leaf thickness is 30% (n=16) for the young leaves and 41% (n=22) for mature leaves.

The anticlinal epidermis cell wall varies in form: The abaxial side has sinuous walls, whereas the adaxial epidermis has straighter walls and the epidermis cells vary in size (values not further recorded). Stomata are of the anisocytic type. Stomata are more abundant abaxially than adaxially. The cuticle on the epidermis is striated. Early in the vegetation period, these striations run perpendicular to the pores around the stomata (figure online see acknowledgements URL). Later, however, in addition to these alea (win-like striations on the side of a stoma), striations run over the entire periphery of the cuticle (figure online). No trichomes, papillae, hypodermis, double epidermis or crystals were recorded.

Element Analysis (EDXA)

The highest nickel concentration recorded in samples of the 17 May 2006 harvest (which were air dried) was 2.14 dry weight percent (hereafter %) and was found in the mature leaves. The highest average nickel concentration in this harvest was also recorded in the mature leaves (0.94% ± 0.05). Interestingly, nickel was absent in the young leaves and inflorescence axes. Zinc was distributed evenly over the organs with the exception of the mature leaves (see table 2 & figure 2). In the mature leaves, nickel was concentrated in the epidermis. Comparing the nickel concentration in the parenchyma (0.18% ± 0.02) with that of the adaxial epidermis reveals a significant ($P<0.05$) difference. Not significant ($P<0.20$) is the difference between the abaxial (0.40% ± 0.02) and adaxial epidermis (0.52% ± 0.02). No nickel was found in the vascular bundles. Due to the low number of

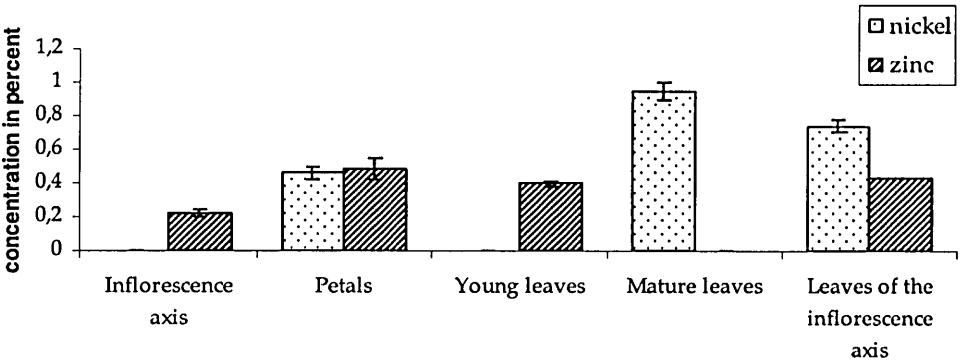

Fig. 2: Nickel and zinc concentration (dry weight percentage) in the air-dried organs of *T. goesingense*; standard error (harvest 17 May). – Nickel und Zink-Konzentration (%TG) in luftgetrockneten Organen von *T. goesingense*; Standardabweichung (Ernte 17. Mai).

observations (n = 2), these significance levels are doubtful. The zinc concentration was higher in the mesophyll (0.46% ± 0.2) than in the epidermis (0.10% ± 0.01 $P<0.20$).

Examination of the plants that were harvested one week later (24 May) and treated with the freeze-drying technique revealed higher nickel concentrations (table 2) but similar zinc concentrations (data not shown). Interestingly, the leaves of the inflorescence axis contained more nickel (2.36% ± 0.14) than the mature leaves (2.14% ± 0.08). Moreover, the nickel and zinc concentrations rose in elevated parts of the inflorescence axis. Zinc was again consistently distributed over the organs, even though in this harvest it was present in the mature leaves but not in the young leaves.

In the leaves of the inflorescence axis, nickel was concentrated in the abaxial epidermis (1.24% ± 0.06) compared to the adaxial epidermis (0.62% ± 0.02). In the vascular bundles, no nickel contents were recorded, and in the parenchyma the concentration was only one tenth that in the abaxial epidermis. Zinc was also equally distributed in the leaves of the inflorescence axis (differences not significant expect for the difference between the abaxial epidermis and the parenchyma ($P<0.001$)). In the young and mature leaves, nickel was compartmentalised in the epidermis. Zinc was again more equally distributed between the tissues. Samples from the 4 July harvest show a totally different tendency than samples from other dates (table 2 & figure 3). The nickel content in the mature leaves (0.25% ± 0.18) was lower than that of the young leaves (0.38% ± 0.04). In contrast to the previous two harvests, no nickel was recorded in the higher leaves.

Zinc was not equally dispersed: leaves of the inflorescence axis (0.67% ± 0.02) and mature leaves (0.54% ± 0.03) differed significantly from young leaves (0.08% ± 0.01) (inflorescence leaves $P<0.01$, mature leaves $P<0.0001$). No significant difference was found when the mature and inflorescence axis leaves were compared to the lower inflorescence axes.

In the young leaves, nickel was concentrated in the epidermis cells (0.73% ± 0.26). A significant difference ($P<0.05$) between values in the epidermis cells and mesophyll cells (0.08% ± 0.04) was detected. The mature leaves showed no significant difference between the mesophyll and epidermis. In the lower inflorescence axis, zinc was equally distributed between the mesophyll (with vascular bundles) and the epidermis ($P<0.50$). This was also the case between the mesophyll and epidermis of mature leaves ($P<0.10$).

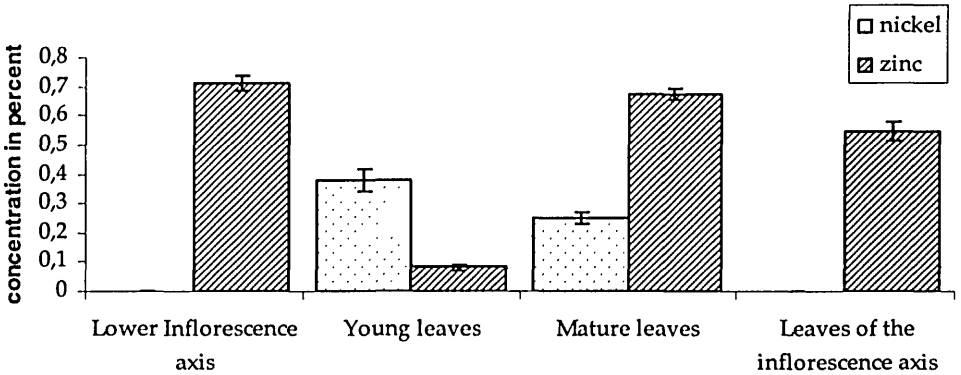

Fig. 3: Nickel and zinc concentration in dry weight percentage of freeze-dried organs of *T. goesingense;* standard error (harvest 4 July). – Nickel und Zink Konzentration (%TG) in gefriergetrockneten Organen von *T. goesingense*; Standardabweichung (Ernte 4. Juli).

The harvest on 17 August contained only the rosette leaves, so that only these could be analysed. Generally, concentrations were lower than those of previous harvests.

In the mesophyll of mature leaves, neither nickel nor zinc were detected: all of the recorded metals were found in the epidermis cells (nickel significant $P<0.05$, zinc not significant $P<0.10$). The mesophyll of young leaves contained no nickel. No significant difference was found between the mesophyll and the epidermis($P<0.20$). Like in the previous harvest, that of 2 October yielded only rosette leaves. Zinc was found only in young, not in mature leaves, the reverse of the last harvest (17-08-06). The nickel concentration in the mature leaves was higher than that in the young leaves, but this was not significant (table 2).

In both mature and young leaves, nickel was restricted to the epidermis cells. The average values in the former were $0.29\% \pm 0.11$, in the latter $0.07\% \pm 0.05$. No nickel was recorded in the mesophyll; this was a significant ($P<0.05$) difference when compared to the epidermis in the mature leaves, but not significant ($P<0.50$) for the young leaves.

The material of the last harvest (28 November) was again air dried in a desiccator. This harvest was conducted after the plants were moved from the outdoor location into the greenhouse. New organs had emerged between the rosette leaves. Although SEM images did not clarify their nature, indications point towards the beginning of a new flowering period. Interestingly, the nickel and zinc concentrations were higher compared to the previous two harvests.

The mature leaves again showed a higher nickel concentration than the young leaves. Zinc was found in all organs (see table 2 and figure 4). In contrast to the last two harvests, nickel also occurred in the mesophyll ($0.33\% \pm 0.10$). In the mature leaves, the nickel concentration in the epidermis ($0.75\% \pm 0.32$) was still higher than in the mesophyll. In the mature leaves, this difference was not significant ($P < 0.50$). In the young leaves, the difference between epidermis ($0.33\% \pm 0.02$) and mesophyll ($0.12\% \pm 0.05$) was significant ($P<0.01$). Nickel was also recorded in the vascular bundles, but due to the small number of measurements no test of significance was performed. Zinc concentrations in the vascular bundle ($0.36\% \pm 0.24$), mesophyll ($0.33\% \pm 0.02$) and epidermis cells ($0.33\% \pm 0.02$) in the young leaves were almost the same, i.e. the differences were not significant (in all cases $P>0.50$).

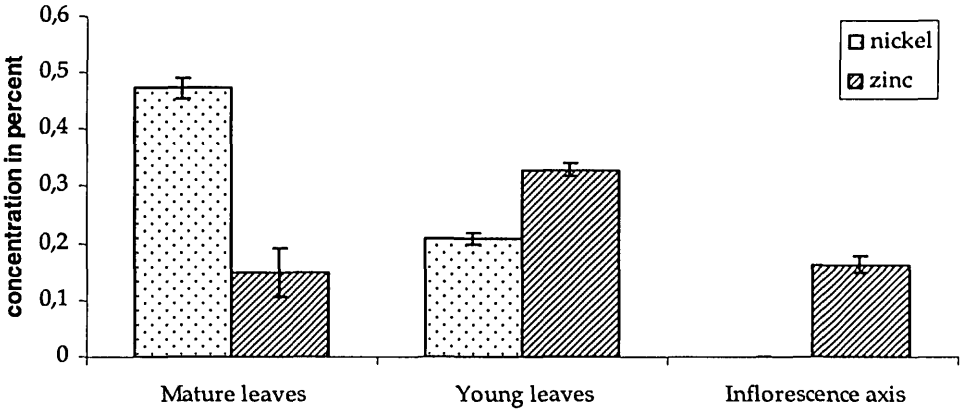

Fig. 4: Nickel and zinc concentration in dry weight percentage in the air dried organs of *T. goesingense*; standard error (harvest 28 November). – Nickel und Zink Konzentration (%TG) in luftgetrockneten Organen von *T. goesingense*; Standardabweichung (Ernte 28. November).

Discussion

The large, elongated epidermis cells recorded previously (Küpper et al. 2001) and in this study apparently are a modification for hyperaccumulation. The mature leaves contain significantly more of these cells than young leaves. In all but one harvest, the latter had lower nickel concentrations in their epidermis than the former. The second indication that such cells are a modification is that their number (and the nickel concentration) did not differ significantly between the abaxial and adaxial epidermis.

An interesting result is the relatively few elongated cells in the leaves of the inflorescence axes and the lack of a difference in the number of elongated cells between the abaxial and adaxial epidermis – nickel, however, was unevenly distributed between the abaxial and adaxial epidermis. One explanation is the temporary function of this organ, which is discarded by the plant body after flowering. The few large, elongated cells recorded here and the high nickel concentrations confirm that these cells do not play a key role in these leaves.

The observed cuticular striations have been interpreted to be an artefact due to drying. The fact that such striations were recorded in almost all harvests and leaves, and that they are not uncommon, makes this explanation unlikely. The second explanation is that water, because of its high surface tension, can only contact the exterior portions of the striations: the small contact area causes water to roll off the leaf. Fungi spores that are also only weakly connected with the striations are then also washed off. This interpretation appears to be valid for *T. goesingense*. Freeman et al. (2005) reported that *T. goesingense* is susceptible to the mildew *Erysiphe cruciferarum* when nickel concentrations are low. Our observation that striations increase during the vegetation period correlates with the decrease in nickel. This would support the Pathogen/Herbivore-Defence-Hypothesis. At elevated nickel concentrations, *T. goesingense* has a resistance to *Erysiphe cruciferarum*. When the concentrations decline, however, susceptibility increases and secondary cuticular striations increase. This represents an alternative to an elevated nickel concentration in resisting mildews. The anisocytic stomata are a family characteristic of the Brassicaceae and are also visible in photos of *T. minimum* made by Aigner (2005).

This is not the first report of nickel in petals (SEVERNE & BROOKS 1972, JAFFRÉ & SCHMID 1974, REEVES et al. 1981), but is the first study to report nickel in the petals of *T. goesingense*. Previously reported nickel contents are 1266 mg kg^{-1} in seeds (REEVES & BAKER 1984) and > 1000 mg kg^{-1} nickel in the fruits of *T. goesingense* (AIGNER 2005).

Our study directly confirms the disposal of the Plant-Body-Hypothesis: the leaves of the inflorescence axes accumulate abundant nickel and these leaves are discarded after flowering. This is supported by the dramatic loss of nickel and zinc after the flowering period. The elevated metal concentrations early in the vegetation period can be attributed to the long time available to accumulate the metals or to the extensive plant growth prior to flowering and the additional nutrient uptake here. It is unlikely that metals could be lost in another way because no trichomes or other adaptations were recorded.

The nickel concentration increased again in the last harvest. This can be explained by the relocation of the plants from their outdoor location to a greenhouse. This relocation simulated the start of a new season and prompted renewed growth. Growth releases extra root exudates, which mobilize heavy metals. These are then deliberately taken up (enhanced ability to Transport-Metals Theory) or taken up as a by-product with valuable nutrition (Inadvertent-Uptake-Theory). Not tested in this study, and not mentioned in the literature on *T. goesingense,* is an alternative detoxification pathway in which nickel might bind to chemical complexes and thus become deactivated (SASSE 1976).

Future research might focus on measuring the glucosinolate (a secondary metabolite for defence) concentration of *T. goesingense*. Plants of the family Brassicaceae that contain sinigrin are know to be less susceptible to *Erysiphe cruciferarum* (MENARD et al. 1999): increasing glucosinolate concentrations when metal concentrations drop would provide support for the Pathogen/Herbivore-Defence-Hypothesis. In addition, water potential could be recorded (R. MAIER, personal communication) to test the drought resistance theory. Accordingly, at high metal concentrations the water potential would be expected to increase .

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