Plant Physiol. (1997) 115: 1641–1650

The Role of Metal Transport and Tolerance in Nickel Hyperaccumulation by *Thlaspi goesingense* Hálácsy[1]

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Metal hyperaccumulators are plants that are capable of extracting metals from the soil and accumulating them to extraordinary concentrations in aboveground tissues (greater than 0.1% dry biomass Ni or Co or greater than 1% dry biomass Zn or Mn). Approximately 400 hyperaccumulator species have been identified, according to the analysis of field-collected specimens. Metal hyperaccumulators are interesting model organisms to study for the development of a phytoremediation technology, the use of plants to remove pollutant metals from soils. However, little is known about the molecular, biochemical, and physiological processes that result in the hyperaccumulator phenotype. We investigated the role of Ni tolerance and transport in Ni hyperaccumulation by *Thlaspi goesingense*, using plant biomass production, evapotranspiration, and protoplast viability assays, and by following short- and long-term uptake of Ni into roots and shoots. As long as both species (*T. goesingense* and *Thlaspi arvense*) were unaffected by Ni toxicity, the rates of Ni translocation from roots to shoots were the same in both the hyper- and nonaccumulator species. Our data suggest that Ni tolerance is sufficient to explain the Ni hyperaccumulator phenotype observed in hydroponically cultured *T. goesingense* when compared with the Ni-sensitive nonhyperaccumulator *T. arvense*.

The Ni requirement of plants is generally very low, 1.7 nmol g^{-1} Ni or less in tissue dry biomass (Brown et al., 1988; Dalton et al., 1988). Symptoms of Ni toxicity can be observed between 0.19 and 0.85 μmol g^{-1} Ni in plant dry biomass. These symptoms include the inhibition of root elongation and interveinal chlorosis, the latter possibly a consequence of the interference of Ni with chlorophyll formation (Woolhouse, 1983; Gabbrielli et al., 1990; Brune and Dietz, 1995; Marschner, 1995). The majority of plants that occur on metalliferous soils are known to exclude toxic metals from their shoots (Baker and Walker, 1990). A number of species, however, have developed an unusual adaptation to metal-rich soils. Instead of excluding toxic metals, so-called hyperaccumulators accumulate metals such as Ni, Zn, or Co in their aboveground biomass. This trait is highly attractive for the development of technologies aimed at the decontamination of metal-polluted soils using plants (Chaney, 1983; Salt et al., 1995). Ni concentrations in field-collected leaves of Ni hyperaccumulators are greater than 1000 parts per million (17 μmol g^{-1}) in dry biomass, and concentrations as high as 650 μmol g^{-1} Ni in dry biomass have been reported (Baker and Brooks, 1989; Reeves, 1992). Ni concentrations in the surrounding vegetation usually range between 5 nmol g^{-1} and 1.7 μmol g^{-1} (Brooks and Dudley, 1987). Metal hyperaccumulation may protect plants against herbivory and attack by fungal and bacterial pathogens (Boyd and Martens, 1994; Boyd et al., 1994; Pollard and Baker, 1997).

The high Ni concentrations present in the leaves of a hyperaccumulator suggest the existence of a detoxification system for the metal within the plant. Hyperaccumulator species in the genus *Alyssum* have been reported to be more Ni tolerant than a nonhyperaccumulator species originating from the same metalliferous soil (Gabbrielli et al., 1990), and more tolerant than a related species from a nonmetalliferous habitat (Homer et al., 1991; Rauser, 1995). Among the compounds that have been proposed to participate in metal detoxification in hyperaccumulators are low-molecular-weight chelators such as citrate (Lee et al., 1978) and free His (Krämer et al., 1996).

To account for the high metal concentrations in the shoots of hyperaccumulators it has been suggested that hyperaccumulators may take up metals from the soil solution at an elevated rate or that they have an increased ability to translocate metals into the shoots (Brown et al., 1995; Lasat et al., 1996). However, the relationship between transport rates of Ni into the root and within the plant and the high concentrations of Ni detected in the leaves of field-grown hyperaccumulators is still poorly understood.

Thlaspi goesingense Hálácsy occurs on Austrian Ni-rich ultramafic (serpentine) soils, where it has been reported to hyperaccumulate Ni (Reeves and Brooks, 1983). An ecotype from a calcareous soil is also able to hyperaccumulate Ni when grown on an ultramafic substrate (Reeves and Baker, 1984).

We investigated Ni tolerance and uptake into roots and shoots in *T. goesingense* and a weedy population of the

[1] This research was supported by a North American Treaty Organization fellowship awarded to U.K. by the German Academic Exchange Service, by the U.S. Department of Energy (grant no. DE-FG07-96ER20251) to D.E.S., and by Phytotech to I.R.

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Abbreviations: ANOVA, analysis of variance; ICP, inductively coupled plasma-emission spectroscopy.

related nonaccumulator *Thlaspi arvense* L. Our aim was to assess the roles of metal tolerance and transport in the hyperaccumulator phenotype by comparing *T. goesingense* with *T. arvense*. To our knowledge, this is the first attempt to investigate short-term Ni uptake and root-to-shoot translocation rates in a Ni hyperaccumulator.

MATERIALS AND METHODS

Seed Material and Analysis of Field-Collected Plant and Soil Samples

Seeds of *Thlaspi arvense* L. were obtained from the Crucifer Genetics Cooperative (University of Wisconsin, Madison). Seeds of *Thlaspi goesingense* Hálácsy were collected from plants growing on an ultramafic soil in Redschlag, Austria. Leaf samples collected from randomly selected individuals of *T. goesingense* and other species, as well as soil samples, were analyzed on site in the same ultramafic area. Soil samples were collected in the immediate vicinity of the roots of the plants used for leaf analysis. Plant samples were rinsed, blotted with absorbent paper, and gently dried using a portable gas stove. Dried plant material and dry soil samples were placed in plastic vials with mylar windows, and elemental contents were determined using a portable x-ray fluorescence analyzer (Spectrace 9000, TN Technologies, Round Rock, TX).

Instrument readings were verified daily by running standards of a known composition, which had been previously determined by ICP. X-ray fluorescence analysis gave readings between 77 and 78% of those determined by ICP on identical samples, irrespective of both the plant species the material was taken from and the Ni concentration therein. For ICP analysis (Fisons Accuris, Fisons Instruments, Beverly, MA), the air-dried plant material was digested in 5 mL of concentrated HNO_3 (trace metal grade) at 180°C until the remaining volume of digest solution was less than 2.5 mL, and then digests were clarified by adding 1 mL of $HClO_4$ and heating to 180°C. Certified National Institute of Standards and Technology plant (peach leaf) standards were carried through the digestion protocol and analyzed as part of the quality assurance/quality control program. Reagent blanks and spikes were used where appropriate to ensure accuracy and precision in the analysis.

Pre-Experimental Plant Growth

If not stated otherwise, seeds were germinated on filter paper moistened with 2.8 mmol L^{-1} $Ca(NO_3)_2$ for 1 week. Subsequently, 30 seedlings were transferred into 12 L of hydroponic solution. Seedlings were initially supported by moist vermiculite and later by cotton wool. Solutions were continuously aerated and exchanged at intervals of 18 to 7 d, according to plant size. Since growth rates of *T. goesingense* were substantially lower than those of *T. arvense*, experiments were performed using plants with equivalent numbers of leaves. The composition of all hydroponic solutions was as follows: 0.28 mmol L^{-1} Ca^{2+}, 0.6 mmol L^{-1} K^+, 0.2 mmol L^{-1} Mg^{2+}, 0.1 mmol L^{-1} NH_4^+, 1.16 mmol L^{-1} NO_3^-, 0.1 mmol L^{-1} $H_2PO_4^-$, 0.2 mmol L^{-1} SO_4^{2-}, 4.75 μmol L^{-1} ferric tartrate, 0.03 μmol L^{-1} Cu^{2+}, 0.08 μmol L^{-1} Zn^{2+}, 0.5 μmol L^{-1} Mn^{2+}, 4.6 μmol L^{-1} H_3BO_3, and 0.01 μmol L^{-1} MoO_3. Plants were cultivated in a growth chamber with 10-h light periods, with light provided by fluorescent and incandescent lamps at a light intensity of 20,800 lux. All plants were maintained at day/night temperatures of 22/22°C and a constant humidity of 50%.

Determination of Ni Tolerance and Concentration-Dependent Ni Uptake

Plant roots were grown axenically. Seeds were surface sterilized by rinsing in 75% ethanol for 5 min followed by incubation in 2.6% (w/v) sodium hypochlorite containing 0.01% (v/v) Triton X-100 as a surfactant for 15 min. After four washes in sterile, deionized water, seeds were transferred onto agar plates containing 1 mmol L^{-1} $Ca(NO_3)_2$, 2 mmol L^{-1} KH_2PO_4, 4 mmol L^{-1} KNO_3, 0.3 mmol L^{-1} $MgSO_4$, 0.18 mmol L^{-1} $FeCl_3$, 42.26 μmol L^{-1} H_3BO_3, 0.312 μmol L^{-1} $CuSO_4$, 9.10 μmol L^{-1} $MnCl_2$, 0.106 μmol L^{-1} MoO_3, 0.765 μmol L^{-1} $ZnSO_4$, 3% (w/v) Suc, and 1.2% (w/v) agarose (pH 6.0–6.5), and germinated in the dark at 22°C for 1 week. The etiolated seedlings were transferred individually into small glass vials containing 10 mL of sterile hydroponic solution. Soft Styrofoam stoppers used to cap the vials were incised radially to provide support for the hypocotyls. Solutions were exchanged weekly and the vials were gently agitated on a rotary shaker (Lab-Line Instruments, Melrose Park, IL) at 60 rpm to provide aeration and mixing. Growth conditions were as described above. Light intensity was measured at 17,200 lux.

Four-week-old *T. goesingense* and 2-week-old *T. arvense* of uniform sizes (five replicate plants, each in one individual vial, for each concentration) were exposed to various concentrations of Ni, added as $Ni(NO_3)_2$ to the sterile hydroponic solution, composed as described above (containing 0, 10, 25, 50, 75, and 100 μM $Ni[NO_3]_2$ for *T. arvense* and 0, 10, 50, 100, 250, and 500 μM $Ni[NO_3]_2$ for *T. goesingense*). Solutions were exchanged daily. Exposure to toxic metals can affect plant-water relations (Barceló and Poschenrieder, 1990). Among the toxic effects of Ni is stomatal closure, which may lead to a reduction in transpirational water loss (Rauser and Dumbroff, 1981). Vials were weighed before and after the exchange of solutions to determine water loss. To measure plant evapotranspiration, the daily water loss from vials containing solution but no plants was subtracted from the total daily water loss from vials containing both plants and solution. The resulting values corresponded to the total daily water loss from the plant through transpiration and evaporation, reduced by the daily gain in plant fresh biomass. Since the daily gain in plant fresh biomass proved to be small compared with plant water loss, this was a valuable technique for assessing Ni toxicity repeatedly in a noninvasive manner over a period of time.

After 7 d, plants were harvested, separated into roots and shoots, rinsed in deionized water, blotted, dried at

70°C for 3 d, and subsequently weighed. The gain in dry biomass was determined by subtracting the initial dry biomass determined for roots and shoots of each species (average of 10 plants) sampled at the start of the experiment.

For the analysis of Ni, 5 mL of concentrated HNO_3 was added to each sample of dried plant material in acid-washed Pyrex test tubes, and samples were digested in a heating block at 180°C until the remaining solutions appeared clear. Digests were decolorized by adding 1 mL of 30% (w/v) H_2O_2 and heating to 120°C until effervescence ceased. Digests were made up to 12.5 mL with ultrapure water, and Ni concentrations were analyzed by ICP as described above.

Protoplast Assays

Leaves (nos. 4, 5, 6, and 7, counted from the top-emerging leaf downward) were harvested 2 h after the onset of the light period, 22 and 47 d after the start of imbibition for germination for *T. arvense* and *T. goesingense*, respectively (growth conditions were as described in "Pre-Experimental Plant Growth"). The lower epidermis was removed from the leaves of *T. goesingense*. Leaves of both species were feathered by applying parallel cuts (1 mm distance) from the sides of the midribs to the edges of the leaves with a razor blade. For digestion, the feathered leaves were placed with the abaxial side down in Petri dishes containing 10 mL of 400 mM mannitol, 1 mM $CaCl_2$, 5 mM Mes-NaOH (pH 6.0), 0.05% (w/v) BSA, 0.05% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan), and 2% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Industry, Tokyo, Japan), maintained in the light (1600 lux) at 28°C for 3 h with gentle shaking at 45 rpm. The digest was filtered through a 114-μm nylon mesh, and the remainder was rinsed twice with 2.5 mL of wash medium (400 mM mannitol and 1 mM $CaCl_2$).

The resulting suspension was layered onto 2 mL of 15% (w/v) Ficoll (type 400) containing 400 mM mannitol and centrifuged for 10 min at 250*g* using a swinging-bucket rotor. Protoplasts were collected at the Ficoll interface and resuspended in 4 mL of 15% (w/v) Ficoll in 400 mM mannitol, which was then overlaid with 4 mL of 12.5% (w/v) Ficoll in 400 mM mannitol and subsequently with 1 mL of wash medium. The gradient was centrifuged at 250*g* for 20 min, and purified protoplasts were collected at the 12.5/0% Ficoll interface. Protoplasts were resuspended in 10 mL of wash medium. After the sample was centrifuged at 250*g* for 5 min, the supernatant was removed and the protoplasts were suspended in a small volume of wash medium. The quality of the protoplasts was checked microscopically, and protoplasts were counted using a hemacytometer.

For the tolerance assay, approximately 15,000 protoplasts were suspended in a total volume of 1 mL of medium containing 400 mM mannitol and 1 mM $CaCl_2$ in the absence (controls) or in the presence of 250 μM $NiSO_4$ in a multiwell Petri dish 6 h after harvesting the leaves. Conductivity measurements were used to determine the chemical activity of 250 μM $NiSO_4$ in 400 mM mannitol, which was determined to be 81% of the chemical activity of the same concentration of $NiSO_4$ in ultrapure water (Evangelou and Wagner, 1987). Protoplasts were incubated in constant light (1600 lux) at 28°C with gentle shaking at 45 rpm. After 0 and 24 h, aliquots of approximately 500 μL were sampled from individual wells using a pipette tip with a wide opening. Two replicate samples were taken of each Ni treatment and of the controls. Samples were mixed with 6 μL of a 1 mg mL^{-1} solution of fluorescein diacetate in acetone (Larkin, 1976). After 5 min the stained protoplast suspension was transferred onto a hemacytometer and examined under the fluorescence microscope using a blue filter (excitation window 420–490 nm, dichroic mirror 510 nm, and barrier filter 520 nm). One color-slide photograph was taken of an area of 5.1 mm^2 (containing 1.02 μL of protoplast suspension) of each of the four hemacytometer chambers. The developed slides were projected onto a white board and viable protoplasts (appearing bright green) were counted.

Short-Term Time Course of Ni Uptake Using the Isotope ^{63}Ni

Plants were germinated and grown as described in "Pre-Experimental Plant Growth" and were transferred in groups of four into pots of 400 mL of hydroponic solution containing 10 μmol L^{-1} $Ni(NO_3)_2$ and $^{63}NiCl_2$ (0.135 μCi L^{-1}) 39 d after the start of germination for *T. goesingense* and 16 d after the start of germination for *T. arvense*. During Ni exposure plants were illuminated at a light intensity of 7400 lux. At each time (after 2, 4, 6, 8, 10, and 12 h) one plant was removed from each of five replicate pots and roots and shoots were separated. For desorption of ^{63}Ni ions adsorbed to the cell walls, roots were placed into solutions containing 5 mmol L^{-1} $Ca(NO_3)_2$ and 10 μmol L^{-1} $Ni(NO_3)_2$ at room temperature for 20 min (pH between 5.6 and 5.9). This was the optimum desorption time as determined in separate experiments (data not shown). Uptake and desorption solutions were continuously aerated.

Shoots were rinsed in deionized water, and roots and shoots were blotted onto absorbent tissue paper. Plant tissue was dried at 70°C for 3 d and weighed. The tissue was placed in small glass test tubes, crushed with a glass rod, and digested prior to liquid-scintillation counting (modified after Cataldo et al., 1978). For digestion, 200 μL of concentrated HNO_3 was added, and the samples were heated slowly to 150°C in a heating block (2 h at 45°C, 2 h at 60°C, and 4 h at 85°C) and kept at that temperature for 4 h to solubilize the tissue. To remove the remaining color, 100 μL of 50% (w/v) H_2O_2 was added to the digests after cooling below 50°C, and samples were heated gradually to 150°C and were maintained at this temperature until effervescence ceased. This decolorization process was repeated twice. Subsequently, the digests were transferred into glass scintillation vials, and 10 mL of scintillation cocktail (Ready Safe) was added to each sample. ^{63}Ni was determined in a liquid-scintillation counter (LS 5000 TD, Beckman) with a window setting of 0 to 560 kV. Counting efficiencies were 66 to 85% and quench numbers were between 130 and 204. Samples of the experimental solutions were taken before

and after the experiment, and there was no significant depletion of Ni during the experimental period.

Six-Day Time Course of Ni Uptake

For each of the two species, plants of equivalent size (34 and 16 d after the start of germination for *T. goesingense* and *T. arvense*, respectively), were transferred in groups of seven into three pots, each containing 12 L of hydroponic solution amended with 10 μmol L^{-1} $Ni(NO_3)_2$. Solutions were replaced once after 3 d and growth conditions were as described in "Pre-Experimental Plant Growth"). After 0, 1, 2, 3, 4, 5, and 6 d, three replicate plants were harvested (one from each of the three containers). Shoots and roots were separated, and roots were desorbed in 5 mmol L^{-1} $Ca(NO_3)_2$ at room temperature for 20 min. Uptake and desorption solutions were continuously aerated. Both roots and shoots were briefly rinsed in deionized water, blotted, dried, and weighed. The dried plant material was digested in 5 mL of concentrated HNO_3 as described above, and digests were clarified by adding 1 mL of $HClO_4$ and heating to 180°C. Analysis for Ni was performed by ICP as described above.

RESULTS

Ni Hyperaccumulation in *T. goesingense*

Leaves of *T. goesingense* were collected from plants growing in Redschlag, Austria, an ultramafic site. A portable x-ray fluorescence analyzer was used to determine elemental contents of leaves and soils immediately adjacent to the roots of individual plants on site. Ni concentrations in these leaves were 93.3 ± 16.8 μmol Ni g^{-1} dry biomass (as average ± SE; n = 6), more than 3 times higher than the Ni concentrations measured in the soil surrounding the roots of these plants, 24.5 ± 2.4 μmol Ni g^{-1} (as average ± SE; n = 6). It is therefore unlikely that the Ni concentrations detected in the leaf tissue were an artifact due to contamination by soil particles. Leaves of nonaccumulator species, including *Silene cucubalus*, *Rumex* sp., *Genista* sp., and *Galium* sp., contained 4.3 ± 2.5 μmol Ni g^{-1} dry biomass (as average ± SE; n = 4), with rhizospheric Ni concentrations of 11.7 ± 1.2 μmol Ni g^{-1} (as average ± SE; n = 4). Thus, Ni concentrations determined in the leaves of *T. goesingense* were at least 1 order of magnitude higher than those in leaves of the surrounding nonaccumulators, confirming the hyperaccumulator status of *T. goesingense*.

To obtain quantitative data concerning the hyperaccumulator phenotype of *T. goesingense* under controlled experimental conditions, we compared Ni uptake and tolerance of this species with the related nonhyperaccumulator *T. arvense* under exposure to increasing concentrations of Ni nitrate in hydroponic culture (Figs. 1-3). At the end of an exposure period of 7 d, Ni concentrations in shoots and roots differed markedly between the two species (Fig. 1). Shoot Ni concentrations in *T. goesingense* ranged between 13.9 and 113.2 μmol Ni g^{-1} dry biomass and were higher than in *T. arvense*, the shoots of which contained 4.6 to 13.3

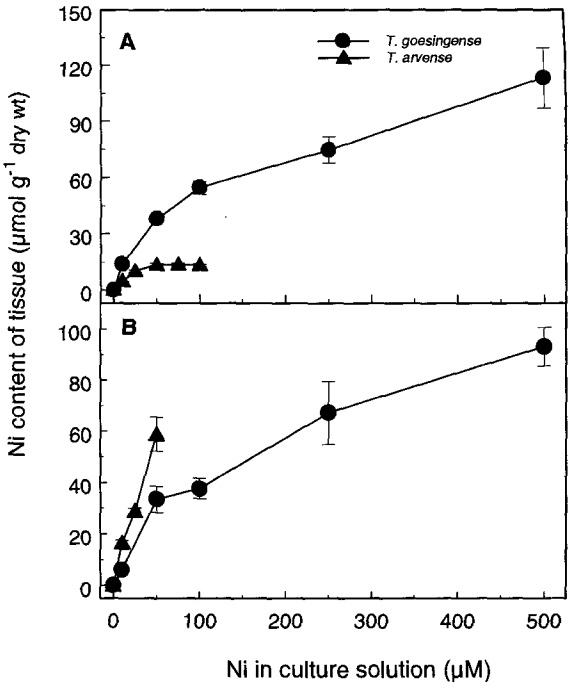

Figure 1. Tissue Ni concentrations in shoots (A) and roots (B) of *T. goesingense* and *T. arvense* as a function of the Ni concentration in the hydroponic solution. Values are means ± SE of five replicate plants harvested after 7 d of exposure to the indicated concentrations of Ni with a daily exchange of solutions. The data presented are from one experiment representative of a total of two independent experiments. Statistical analysis by two-way ANOVA indicated significant differences between Ni concentrations in shoots ($P < 0.001$) and roots ($P < 0.001$) of the two species.

μmol Ni g^{-1} dry biomass (Fig. 1A). For this experiment roots were not desorbed; therefore, the given Ni concentrations include Ni taken up into the root symplast and Ni adsorbed to the root cell walls. In the roots of *T. arvense* Ni concentrations were considerably higher (16.4–58.8 μmol Ni g^{-1} dry biomass) than in the roots of *T. goesingense* (6–33.5 μmol g^{-1}) at any given external Ni concentration in the culture solution at the end of the 7-d-exposure period.

Ni Tolerance

Notable differences in Ni tolerance were observed between *T. goesingense* and *T. arvense* (Figs. 2 and 3). Biomass production of shoots and roots of the non-hyperaccumulator *T. arvense* was reduced to 40% of the unexposed controls by the lowest Ni concentration of 10 μM after 7 d of exposure (Fig. 2). Young leaves of plants affected by Ni toxicity were chlorotic between the veins and developed necrotic lesions. In *T. goesingense* biomass production of neither roots nor shoots was significantly reduced by any of the Ni concentrations used in the experiment, and there were no visible signs of Ni toxicity.

Ni toxicity was detected through a significant reduction in plant water loss from *T. goesingense* only beyond d 6 of exposure to 500 μM Ni (Fig. 3A, see "Materials and Methods"). In *T. arvense* exposure to 100 μM Ni disturbed water

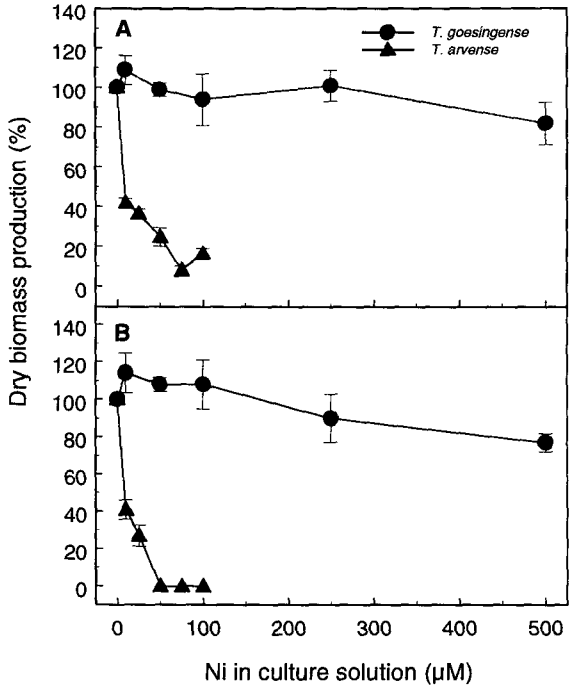

Figure 2. Tolerance was expressed as a percentage of dry biomass production of shoots (A) and roots (B) relative to the respective controls grown in the absence of Ni. Plants were grown and harvested as described in Figure 1. All values are means ± SE of five replicate samples. The data presented are from one experiment representative of a total of two independent experiments. The biomass of *T. goesingense* grown in the absence of Ni was 16.2 ± 1.8 mg (n = 5) and 3.8 ± 0.8 mg (n = 5) for shoots and roots, respectively. The biomass of *T. arvense* control plants grown in the absence of Ni was 27.2 ± 0.6 mg (n = 5) and 4.1 ± 0.3 mg (n = 5) for shoots and roots, respectively. Statistical analysis by one-way ANOVA, which was carried out on logarithmic transforms of the original measurements, indicated that biomass production was not significantly reduced at any Ni concentration in *T. goesingense* but was significantly reduced at the lowest Ni concentration of 10 μM in *T. arvense* ($P < 0.001$).

relations significantly from the 1st d of Ni exposure (Fig. 3B), whereas water relations of *T. goesingense* were unaffected by exposure to the same Ni concentration for a period of 7 d (data not shown). A statistically significant reduction in water loss from *T. arvense* was detected beyond 48 h of exposure to Ni at the lowest Ni concentration of 10 μM (Fig. 3B).

The minimum Ni concentrations in the hydroponic medium that caused Ni toxicity symptoms were about 50-fold higher for *T. goesingense* than for *T. arvense*. Thus, measurement of biomass production and plant water loss showed that *T. goesingense* is considerably more tolerant to Ni than *T. arvense*. Plant water loss appeared to be a more sensitive measurement of metal toxicity than biomass production.

Using a protoplast assay we investigated whether Ni tolerance in *T. goesingense* can also be observed at the cell level (Fig. 4). Upon exposure to Ni for 24 h, the viability of protoplasts isolated from *T. arvense* was significantly reduced to about two-thirds that of the unexposed controls. In *T. goesingense* cell viability of Ni-exposed protoplasts was slightly higher than that of the unexposed controls, suggesting that the protoplasts were more Ni tolerant.

Ni Transport

The higher Ni concentrations found in shoots of *T. goesingense* may be explained by an enhanced rate of Ni transport into the shoots (Fig. 1). To test this we compared short-term Ni uptake in shoots of the two species in a time-course experiment (Fig. 5). The external Ni concentration (10 μM) used in this experiment approximates Ni concentrations found in soil solutions of ultramafic soils (Proctor et al., 1981) and caused no detectable toxicity to the nonhyperaccumulator *T. arvense* within 12 h (Fig. 3 B). In this and all subsequent experiments all roots were desorbed to remove Ni adsorbed to the root cell walls.

Ni accumulation in shoots of *T. goesingense* and *T. arvense* between 0 and 12 h are shown in Figure 5A. Quantitatively, differences in shoot Ni concentrations between the two species were small and did not increase significantly over time, suggesting that accumulation rates in shoots were very similar in both species during the experimental period. This was confirmed statistically using ANOVA and regression analysis. When data were expressed as total Ni

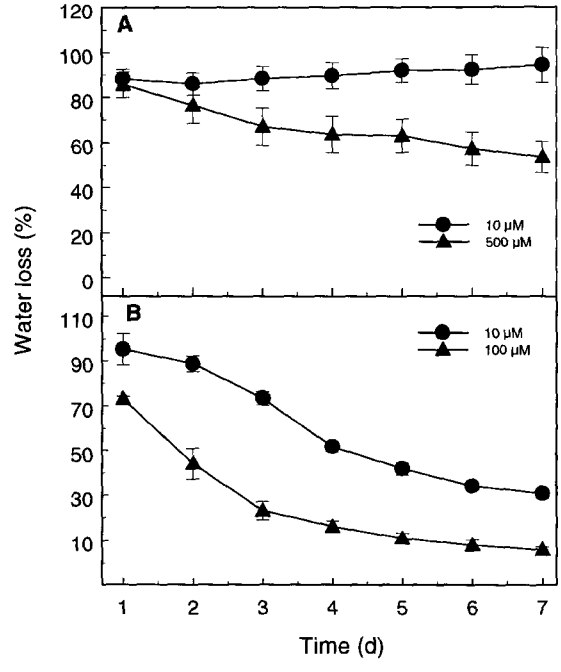

Figure 3. Time course of water loss from *T. goesingense* (A) and *T. arvense* (B) during the first 7 d of exposure to Ni concentrations of 10 and 500 μM (A) or 10 and 100 μM (B). Water loss is expressed as a percentage relative to the controls grown in the absence of Ni. Water loss of the controls increased in a linear fashion from 0.64 ± 0.04 mL on d 1 to 1.22 ± 0.07 mL on d 7 (mean ± SE; n = 5) in *T. goesingense* and from 0.58 ± 0.02 mL on d 1 to 1.97 ± 0.03 mL on d 7 (n = 5) in *T. arvense*. Statistical analysis by one-way ANOVA indicated that water loss was significantly reduced on the 6th d of exposure to a Ni concentration of 500 μM in *T. goesingense* ($P < 0.05$) and on the 3rd and 1st d of exposure to a Ni concentration of 10 and 100 μM, respectively, in *T. arvense* ($P < 0.01$; $P < 0.001$).

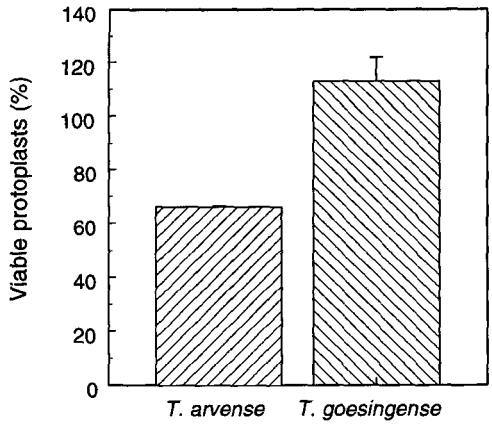

Figure 4. Ni tolerance of leaf protoplasts isolated from *T. arvense* and *T. goesingense*. Given are the percentages of viable protoplasts in an osmotically adjusted solution containing 250 μM Ni^{2+} relative to the controls (0 μM Ni^{2+}) at the end of a 24-h exposure period. Controls contained 104,370 ± 1,342 and 59,937 ± 122 viable protoplasts per milliliter of assay solution for *T. arvense* and *T. goesingense*, respectively. Values are means ± SE of two independent replicates. For each replicate, four chambers were counted, each of which measured approximately 5 mm^2 and contained approximately 1 μL of protoplast suspension. Compared with the control (0 μM Ni) the number of viable protoplasts was significantly reduced at 250 μM Ni in *T. arvense* (Student's *t* test, $P < 0.02$).

translocated to the shoot per unit root biomass, values were the same for both species within the first 6 h and appeared to be slightly higher in *T. arvense* subsequently (Fig. 5B). Root Ni concentrations were higher in *T. arvense* within the uptake period of 12 h (ANOVA; Fig. 6A).

We monitored the uptake of Ni into roots and shoots of both species over a period of 6 d (Figs. 6 B and 7). Root Ni concentrations were significantly higher in *T. arvense* than in *T. goesingense*, and the initial rate of net Ni accumulation was higher in the roots of *T. arvense* than in the roots of the hyperaccumulator *T. goesingense* (ANOVA). In the roots of *T. goesingense* Ni concentrations saturated at 5 μmol Ni g^{-1} dry root biomass after 3 d of exposure to Ni. Ni concentrations in the roots of *T. arvense* increased more rapidly and up to about 14 μmol Ni g^{-1} dry biomass within 4 d, after which no further increase was observed (Fig. 3B).

During a period of 6 d of exposure to Ni, shoot Ni concentrations were slightly higher in *T. goesingense* (Fig. 7A), but statistically no interspecies difference could be detected between rates of Ni accumulation in the shoots (ANOVA, regression analysis). In *T. arvense* shoot Ni concentrations appeared to saturate after 5 d of Ni exposure. At this time Ni toxicity may have affected transport to the shoot (Fig. 3). During a period of 6 d, the total amount of Ni translocated to the shoot per unit root biomass was essentially the same in both species (Fig. 7B). This suggests that Ni concentrations were higher in the shoots of *T. goesingense* than in the shoots of *T. arvense*, because a larger root biomass supplied Ni to a smaller shoot biomass in the hyperaccumulator relative to the nonhyperaccumulator. In fact, the ratio of shoot to root biomass in this experiment was 4.18 ± 0.32 (n = 18) for *T. goesingense* and 7.12 ± 0.63 (n = 18) for *T. arvense*, which supports this interpretation.

DISCUSSION

T. goesingense showed typical characteristics of a metal hyperaccumulator under field conditions, as well as in hydroponic culture under controlled conditions (Figs. 1–3). Ni concentrations between 44 and 211 μmol g^{-1} leaf dry biomass were detected in field populations of *T. goesingense* growing on ultramafic soils, in agreement with both the range of 85 to 256 μmol g^{-1} reported by Reeves and Brooks (1983) and the classification of the species as a Ni hyperaccumulator. We found that *T. goesingense* appeared to grow in spots with slightly higher rhizospheric Ni concentrations than non-accumulator species.

Accumulation of metals to higher concentrations in the shoots than in the roots, as shown here for the hyperaccumulator *T. goesingense* (Fig. 1), has also been reported for *Alyssum* sp. hyperaccumulators (Gabbrielli et al., 1991; Homer et al., 1991; Krämer et al., 1996), including field-collected samples of the Ni hyperaccumulator *Alyssum pintodasilvae* (Menezes de Sequeira and Pinto da Silva, 1991) and of the Zn hyperaccumulator *T. caerulescens* (Baker et al., 1994). Similar to the data concerning *T. goesingense* presented here, biomass production in other metal hyperaccumulators of the genera *Thlaspi* and *Alyssum* were unaffected

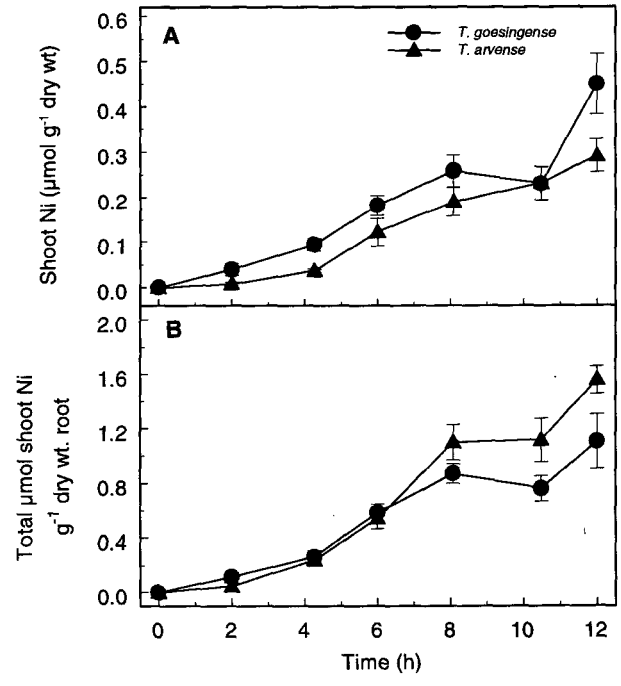

Figure 5. Time course of Ni uptake into shoots of *T. goesingense* and *T. arvense* during the first 12 h of exposure to 10 μM Ni. Ni uptake is expressed as tissue concentration (A) or as root-to-shoot translocation (total amount of shoot Ni taken up per unit root dry biomass [B]). Values are means ± SE of five replicate plants. The data presented are from one experiment representative of a total of three independent experiments covering periods between 12 h and 3 d. Statistical analysis by two-way ANOVA indicated that shoot Ni concentrations were significantly higher ($P < 0.001$) in *T. goesingense* than in *T. arvense*. There was no significant difference between the total amounts of Ni transported to the shoot per unit root dry biomass in the two species.

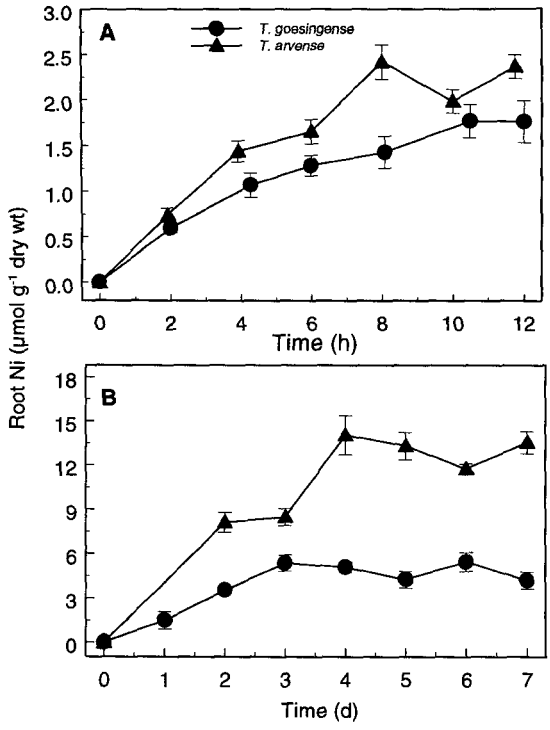

Figure 6. Time course of Ni concentrations in roots of *T. goesingense* and *T. arvense* over 12 h (A) and over 6 d (B). Plants were exposed to 10 µM Ni as described for Figures 5 and 7. Values are means ± SE of five replicate samples. Root Ni concentrations were significantly higher in *T. arvense* ($P < 0.001$) than in *T. goesingense* within both 12 h and 6 d. There was a significant interaction of exposure time with species, indicating a difference in root uptake rate during 6 d of Ni exposure ($P < 0.01$).

by Ni concentrations of up to several hundred micromolar in hydroponic solution (Gabbrielli et al., 1982; Lloyd-Thomas, 1995; Krämer et al., 1996). Compared with the Ni-tolerant *T. goesingense*, *T. arvense* was sensitive to Ni and accumulated higher concentrations in the roots than in the shoots, thus behaving as a nonaccumulator.

Physiologically, the ability of *T. goesingense* to hyperaccumulate Ni on ultramafic soils could be explained by one or several of the following traits: (a) an enhanced ability to mobilize soil-bound Ni in the rhizosphere, (b) the ability to acquire Ni from the soil solution and to transport it to the shoots at a high rate, and (c) tolerance toward high levels of Ni in plant tissues and soil solution. Utilization of a hydroponic culture system to compare *T. goesingense* and *T. arvense* allowed us to investigate the importance of both Ni uptake/translocation and tolerance in the hyperaccumulator phenotype. Ni mobilization in the rhizosphere was not addressed and will be investigated in further studies.

To compare Ni-translocation rates in the two *Thlaspi* spp., short-term (12 h) Ni-exposure periods were used, conditions that did not cause any measurable toxicity symptoms in either species (Fig. 3). Ni concentrations in the shoots were found to be slightly higher in the hyperaccumulator *T. goesingense* than in the nonaccumulator *T. arvense* (Fig. 5A). Slightly elevated Ni accumulation in shoots of *T. goesingense* was also observed over a longer uptake period of 6 d (Fig. 7A). However, when root-to-shoot translocation of Ni was calculated as the total amount of Ni in the shoot normalized to root biomass (Davenport et al., 1997), the resulting net root-to-shoot Ni translocation appeared very similar in both species during the first 4 to 5 d of exposure. Subsequently, Ni toxicity in the nonaccumulator *T. arvense* may have caused Ni translocation to stall (Figs. 3, 5B, and 7B). These data demonstrate that enhanced rates of root to shoot Ni transport are not responsible for the ability of *T. goesingense* to hyperaccumulate Ni.

Studying root and shoot uptake of Zn in the related Zn hyperaccumulator *T. caerulescens* J&C Presl. and *T. arvense*, Lasat et al. (1996) found a higher rate of root uptake and some indication of a significantly increased rate of root-to-shoot transport of Zn in the hyperaccumulator *T. caerulescens*, compared with the nonaccumulator *T. arvense*. The results presented here may indicate that there are differences between Zn and Ni transport in metal-hyperaccumulating *Thlaspi* species. Alternatively, the non-hyperaccumulator and control species *T. arvense* may transport Zn and Ni at different rates. Zn requirement by plants is much higher than Ni requirement (Brown et al., 1988;

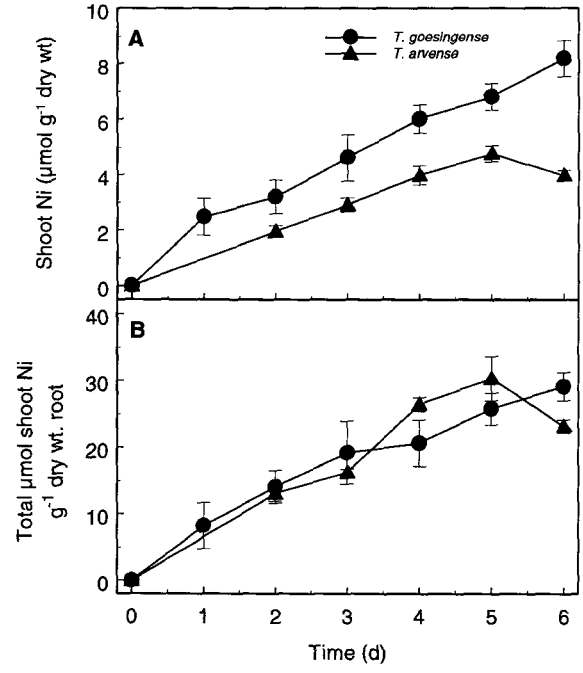

Figure 7. Time course of Ni uptake into shoots of *T. goesingense* and *T. arvense* during the first 6 d of exposure to 10 µM Ni in hydroponic culture. Ni uptake is expressed as tissue concentration (A) or as amount of shoot Ni taken up per unit root dry biomass (B). Values are means ± SE of three replicate plants. The data presented are from one experiment representative of a total of three independent experiments. Statistical analysis by two-way ANOVA indicated that shoot Ni concentrations were significantly higher ($P < 0.001$) in *T. goesingense* than in *T. arvense*. There was no significant difference in the total amounts of Ni transported to the shoot per unit root dry biomass between the two species.

Marschner, 1995). Accidental uptake of Ni may meet the Ni requirement of *T. arvense*; therefore, this species may not have evolved a system to regulate Ni uptake. Zn uptake may be controlled much more tightly, leading to reduced uptake, when Zn supply is sufficient, as in the experiments performed by Lasat et al. (1996), whereas Ni uptake may not be reduced, even under conditions of excess Ni.

Our observation that Ni accumulation in the roots of the nonaccumulator *T. arvense* (Fig. 6) was higher than in the roots of the hyperaccumulator was not the consequence of a reduced export of Ni from root to shoot (Figs. 5B and 7B) but rather suggested an increased net influx into the root symplast of the nonaccumulator.

Accumulation of Ni in the roots of the nonaccumulator *T. arvense* ceases after 4 d of exposure to Ni, which was, like the decline in root-to-shoot Ni transport after 4 to 5 d (Fig. 7B), presumably caused by Ni toxicity. In the roots of the hyperaccumulator *T. goesingense* Ni accumulation also saturated after 3 d. Since this species is tolerant to Ni (Figs. 2, 3A, and 4), we propose that maintaining low symplastic Ni concentrations in the root represents a specific physiological response of the hyperaccumulator to Ni exposure.

We would expect the Ni hyperaccumulator *T. goesingense* to be more tolerant to elevated tissue Ni concentrations than the nonaccumulator *T. arvense*. Measurements of biomass production (Fig. 1), evapotranspiration rates (Fig. 3), and protoplast viability (Fig. 4) confirmed this assumption with respect to Ni concentrations within both the rooting medium and plant tissues. Correlating the onset of Ni toxicity with the ability to transport Ni from the root to the shoot (Figs. 3 and 7), it is clear that the Ni-sensitive nonaccumulator *T. arvense* is unable to maintain efficient root-to-shoot Ni transport after the onset of Ni toxicity. Impaired root-to-shoot transport of magnesium and potassium was also observed in *T. arvense* at this point (data not shown). In contrast, the Ni-tolerant hyperaccumulator species *T. goesingense* is able to resist Ni toxicity and therefore maintain normal rates of root-to-shoot Ni transport. It is this difference in Ni tolerance, reflected in rates of root-to-shoot Ni transport, that mainly explains the differences in Ni accumulation between the two *Thlaspi* spp. observed after 7 d of Ni exposure (Fig. 1), although both species display similar root-to-shoot Ni transport rates under nonphytotoxic conditions.

Because of the central role Ni tolerance plays in the ability of the hyperaccumulator *T. goesingense* to maintain efficient root-to-shoot Ni transport and accumulation, we investigated the basis of this tolerance. The exposure of leaf protoplasts isolated from both *Thlaspi* spp. to Ni demonstrated the existence of a tolerance mechanism operating at the cellular level in the hyperaccumulator *T. goesingense* (Fig. 4). Further investigations are needed to determine how tolerance is achieved.

The evidence presented in this paper suggests that the difference in Ni accumulation observed between hyperaccumulator and non-accumulator *Thlaspi* spp. in hydroponic culture is a consequence of the remarkable Ni tolerance in the hyperaccumulator. Root-to-shoot Ni translocation rates are not elevated in *T. goesingense*. Instead, the Ni sensitivity of the nonaccumulator *T. arvense* causes the breakdown of Ni uptake and therefore reduces long-term accumulation. As a minor factor, a higher root-to-shoot biomass ratio in the hyperaccumulator allows a larger amount of root tissue to load Ni into a relatively smaller amount of shoot tissue, resulting in elevated shoot Ni concentrations.

How do these findings relate to the field observation that shoot Ni concentrations detected in *T. goesingense* are between 1 and 3 orders of magnitude higher than those in surrounding nonaccumulator species? The traits identified here as characteristic of the hyperaccumulator *T. goesingense* are fairly common in the plants found on ultramafic soils: among the vegetation of these nutrient-poor Ni-enriched soils, remarkably large root systems are ubiquitous (Menezes de Sequeira and Pinto da Silva, 1991) and a certain degree of Ni tolerance may be expected in most taxa. Yet, only a very small number of species occurring on ultramafic soils hyperaccumulate Ni (Reeves, 1992).

First, it is possible that the nonaccumulators that occur on ultramafic soils have evolved the ability to exclude Ni from their shoots more effectively than the weedy population of *T. arvense* used in our experiments. This ability would be expected to reduce root-to-shoot Ni-translocation rates compared with the hyperaccumulator species. Metal-tolerant ecotypes of a number of species exclude metals from the shoot more effectively than the sensitive ecotypes. For example, this has been reported for Cu-/Co-tolerant *Silene burchelli*, Zn-tolerant *Silene maritima*, and Cu-tolerant *Silene cucubalus* (Lolkema and Vooijs, 1986; Baker and Walker, 1990).

Second, hyperaccumulators may be able to grow in microenvironments with relatively high bioavailable concentrations of Ni within an ultramafic soil. Gabbrielli et al. (1990) have shown that the Ni-hyperaccumulator species *Alyssum bertolonii* was substantially more tolerant to Ni in hydroponic culture than a population of the nonaccumulator *Silene italica* from the same ultramafic site. Enhanced Ni tolerance may enable *A. bertolonii* to grow in areas containing high available Ni concentrations, whereas other species like *S. italica* may avoid these areas. Comparing serpentine and nonserpentine populations of the nonaccumulator *Silene dioica*, Westerbergh (1994) could not detect any difference in Ni tolerance.

Third, hyperaccumulator roots may have the ability to mobilize poorly available soil metal for plant uptake (Whiting et al., 1997). A number of plants are known to enhance nutrient availability by releasing root exudates (Marschner, 1995). In soil containing nonphytotoxic background levels of metals, we would expect root-to-shoot Ni-transport rates to be equal in hyperaccumulators and nonaccumulators based on the work presented here. However, some hyperaccumulator species are still able to accumulate higher metal concentrations in their shoots than surrounding nonaccumulator plants (Baker et al., 1991; Brown et al., 1994; McGrath et al., 1997). This supports the idea that hyperaccumulators may be able to mobilize soil-bound metals into the soil solution, making them more available for plant uptake. However, unequivocal evidence for such a mechanism remains to be established.

CONCLUSION

Our data suggest that Ni tolerance, and not enhanced rates of Ni transport from root to shoot, is of primary importance in generating the hyperaccumulator phenotype observed in hydroponically cultured *T. goesingense*. The fact that protoplasts isolated from *T. goesingense* were more tolerant than those isolated from *T. arvense* suggests the existence of a cellular mechanism of Ni tolerance in the leaves of the hyperaccumulator. Our future research efforts will address the cellular mechanisms of Ni detoxification in *T. goesingense* and the possible release of Ni-chelating compounds from the roots of *T. goesingense* into the rhizosphere.

ACKNOWLEDGMENTS

We would like to thank Dr. Faith Belanger and Dr. Eric Lam for the kind permission to use their microscopic equipment and Dr. Lee Meisel for her patient, practical introduction to the fluorescence microscopy setup. We also thank Meagan O'Brien for her assistance with plant growth.

Received May 28, 1997; accepted September 8, 1997.
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