

# Influence of the zinc hyperaccumulator *Thlaspi caerulescens* J. & C. Presl. and the nonmetal accumulator *Trifolium pratense* L. on soil microbial populations

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**Abstract:** Metal hyperaccumulator plants like *Thlaspi caerulescens* J. & C. Presl. are used for phytoremediation of contaminated soils. Since little is known about the rhizosphere of hyperaccumulators, the influence of *T. caerulescens* was compared with the effects of *Trifolium pratense* L. on soil microbes. High- and low-metal soils were collected near a zinc smelter in Palmerton, Penn. Soil pH was adjusted to 5.8 and 6.8 by the addition of  $\text{Ca}(\text{OH})_2$ . Liming increased bacterial populations and decreased metal toxicity to levels allowing growth of both plants. The effects of the plants on total (culturable) bacteria, total fungi, as well as cadmium- and zinc-resistant populations were assessed in nonrhizosphere and rhizosphere soil. Both plants increased microbial populations in rhizosphere soil compared with nonrhizosphere soil. Microbial populations were higher in soils planted with *T. pratense*, but higher ratios of metal-resistant bacteria were found in the presence of *T. caerulescens*. We hypothesize that *T. caerulescens* acidifies its rhizosphere. Soil acidification in the rhizosphere of *T. caerulescens* would affect metal uptake by increasing available metals around the roots and consequently, increase the selection for metal-resistant bacteria. Soil acidification may be part of the hyperaccumulation process enhancing metal uptake from soil.

**Key words:** phytoremediation, *Thlaspi caerulescens* J. & C. Presl., *Trifolium pratense* L., rhizosphere, soil microbial populations.

**Résumé :** Des plantes hyper-accumulatrices de métaux comme *Thlaspi caerulescens* J. & C. Presl. sont utilisées pour le phytoremédiation de sols contaminés. Puisque nous en savons peu sur la rhizosphère des hyper-accumulateurs, l'influence de *T. caerulescens* a été comparée aux effets de *Trifolium pratense* L. sur des microbes du sol. Des sols à teneur élevée ou faible en métaux furent recueillis aux abords d'une fonderie de zinc à Palmerton, Penn. Le pH du sol fut ajusté à 5,8 et 6,8 par l'ajout de  $\text{Ca}(\text{OH})_2$ . Le chaulage a augmenté les populations bactériennes et a diminué la toxicité due aux métaux jusqu'à des niveaux permettant la croissance des deux plantes. Les effets des plantes sur les bactéries totales (cultivables), sur les champignons totaux, et sur les populations résistantes au cadmium et au zinc furent évalués dans un sol avec ou sans rhizosphère. Les deux plantes ont augmenté les populations de bactéries comparativement au sol sans rhizosphère. Les populations de microbes étaient supérieures dans des sols peuplés avec *T. pratense*, mais une plus grande proportion de bactéries résistantes aux métaux était observée en présence de *T. caerulescens*. Nous émettons comme hypothèse que *T. caerulescens* acidifie sa rhizosphère. L'acidification du sol dans la rhizosphère de *T. caerulescens* affecterait l'acquisition de métaux en augmentant les métaux libres autour des racines et par conséquent augmenterait la sélection pour les bactéries résistantes aux métaux. L'acidification du sol pourrait faire partie du processus d'hyper-accumulation accentuant l'extraction des métaux du sol.

**Mots clés :** phytoremédiation, *Thlaspi caerulescens* J. & C. Presl., *Trifolium pratense* L., rhizosphère, populations microbiennes du sol.

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Phytoremediation, a new low-cost solution to soil contamination compared with traditional removal and (or) disposal techniques, has been proposed to remove excess metals from

soils (Chaney 1983). This technology uses hyperaccumulator plants that can accumulate in excess of  $10 \text{ g}\cdot\text{kg}^{-1}$  metal in shoots. To date, little information is available on the effects

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**Table 1.** Soil characteristics used for the study of the influence of *Thlaspi caerulescens* and *Trifolium pratense* on microbial populations.

Soil		Mg (kg·ha <sup>-1</sup> )	P <sub>2</sub> O <sub>5</sub> (kg·ha <sup>-1</sup> )	K <sub>2</sub> O (kg·ha <sup>-1</sup> )	Ca (kg·ha <sup>-1</sup> )	NO <sub>3</sub> -N (kg·ha <sup>-1</sup> )	O.M.* (g·kg <sup>-1</sup> )	Sand (g·kg <sup>-1</sup> )	Silt (g·kg <sup>-1</sup> )	Clay (g·kg <sup>-1</sup> )	CEC <sup>†</sup> (cmol·kg <sup>-1</sup> )
High metal – pH 5	16.8	40.3	57.1	73.9	11.2	18.2	440	310	250	3.87	
Low metal – pH 5.8	73.9	36.9	238.6	396.5	11.2	40.6	390	370	240	4.96	

\*Organic matter.

†Cation exchange capacity.

of hyperaccumulators on soil microbial populations. Higher microbial numbers and activities are typically observed in the plant rhizosphere (Lynch and Whipps 1990; Tate 2000), although high soil metal content is known to reduce microbial numbers (Kelly and Tate 1998; Fließbach et al. 1994; Wuertz and Mergeay 1997). Hyperaccumulators, because of their specific capacity to remove metals from soil, may alter metal equilibrium in their rhizosphere, resulting in rhizosphere populations that may be different from the rhizosphere of nonhyperaccumulator plants. Rhizobacteria have been shown to affect the plant's ability to survive in the presence of high levels of metal in soil. Burd et al. (1998, 2000) showed that the presence of a metal-resistant plant growth-promoting bacterium, *Kluyvera ascorbata* SUD 165, isolated from soils enriched in metals, directly affects germination and survival in high-metal soil.

The objective of the current study was to determine how *Thlaspi caerulescens* J. & C. Presl., a zinc (Zn) hyperaccumulator reported to accumulate Zn in aboveground biomass up to a concentration of 3% (Brown et al. 1994 and 1995), modifies microbial populations around its rhizosphere. The effects of *T. caerulescens* on microbial populations were compared with the effects produced by *Trifolium pratense* L. *Trifolium pratense* was selected as a reference species because it was found at one of the studied sites, and it also has a similar root growth pattern to *T. caerulescens*.

Two soils [Klinesville very stoney silt loam (loamy-skeletal, mixed mesic Lithic Dystrudept)] differing in metal content were collected in the vicinity of a Zn smelter in Palmerton, Penn. The vegetation of both sites differed drastically. The low-metal soil supported a healthy flora (maple-beech-birch forest type). The high-metal soil was so toxic that it supported no vegetation. Both soils were collected from the surface horizon (0–15 cm) and sieved through a 4-mm stainless steel sieve to remove rocks and undecomposed organic materials. They were analyzed by the soil-testing laboratory of the University of Maryland (Table 1). Soils were amended to 225 kg N·ha<sup>-1</sup> as NH<sub>4</sub>NO<sub>3</sub> and 225 kg P·ha<sup>-1</sup> as KH<sub>2</sub>PO<sub>4</sub> to ensure that plant growth was not inhibited by lack of nutrients. Soils were stored at 4°C and used within 2 weeks.

Since soil pH is known to affect plant growth, rhizosphere populations, metal availability, and metal uptake, pH was altered by adding Ca(OH)<sub>2</sub> to one-half of each of the soils. A total of five soil-pH combinations were then obtained: high-metal soil – pH 5.0 (no pH adjustment), high-metal soil – pH 5.8, high-metal soil – pH 6.8, low-metal soil – pH 5.8 (no pH adjustment), and low-metal soil – pH 6.8. For each

of the soil-pH combinations, three treatments were examined: unvegetated soil, soil sown with *T. caerulescens*, and soil sown with *T. pratense*.

Prior to seeding, seeds were surface sterilized for 10 min in a solution of 70% ethanol followed by 10 min in a 0.5% solution of Na hypochloride and rinsed three times in sterile distilled water. Fifteen seeds of *T. pratense* were planted on the soil surface and covered with loose soil. Since *T. caerulescens* germinates very slowly, it was grown for 1 month on Pro-Mix® (Premier Horticulture, Dorval, Que. Canada), a soilless potting mix, prior to transplantation into soil (one plant per pot). Day 0 of the experiment was defined as the day of seeding for *T. pratense* and the day of transplantation for *T. caerulescens*. Pots were placed in a growth chamber at 28°C and 60% humidity with a 12-h photoperiod. Pots were watered to 60% of field capacity by addition of sterile distilled water.

At each sampling time, numbers of culturable bacteria, culturable fungi, and cadmium (Cd)- and Zn-resistant bacteria were determined in both unvegetated and rhizosphere soils. Ten grams of soil or the whole soil rhizosphere (defined as roots and soil remaining attached to the roots after separation of bulk soil from the roots by gentle shaking) were blended in sterile distilled water for 1 min at 22 000 rpm in a Waring blender. After allowing sand to settle for 1 min, bacteria were enumerated by the dilution-plating method. Bacteria were grown on RIMm, a modification of the rhizosphere isolation medium developed by Buyer (1995). The RIMm was buffered with 50 mM of 2-(*N*-morpholino)ethanesulfonic acid to a final pH of 6.15, and the absence of iron in RIMm was corrected by adding 10 µM iron chelated with *N,N*-di(2-hydroxybenzyl) ethylenediamine-*n,n*-diacetic dihydrochloride dihydrate (Delorme et al.<sup>2</sup>). Metal-resistant bacteria were assessed on RIMm medium containing either 22 mM of Zn-IDA (iminodiacetic acid) or 500 µM of Cd-IDA. Metals were added as an IDA chelate to maintain the same level of free metal during incubation.

Data were analyzed using SAS-PC v6.12 (SAS Institute Inc., Cary, N.C.). Log-transformed means of the different treatments were separated using the Waller-Duncan *k*-ratio *t* test after it was determined that there was a significant (*P* < 0.05) treatment effect using the general linear model procedure. Data presented are arithmetic means of untransformed data.

Liming strongly decreased available metals, especially for metals extracted with water and calcium nitrate (Brown et al.

<sup>2</sup>Delorme, T.A., Schwartz, C.E., Angle, J.S., and Chaney, R.L. Distribution of bacteria with particle size in soils containing different levels of metal. Soil Sci. Soc. Am. J., submitted for publication.

**Table 2.** Total (hot nitric digestion), diethylenetriamine pentaacetic acid - extractable, calcium-exchangeable, and water-soluble metal concentrations in two metal-contaminated soils with varying pH.

Soil	Cadmium (mg·kg <sup>-1</sup> )				Zinc (mg·kg <sup>-1</sup> )			
	Total	DTPA	Calcium	Water	Total	DTPA	Calcium	Water
High metal – pH 5.0	5.3a	2.1a	1.6a	0.6a	227a	48.0a	38.8a	18.0a
High metal – pH 5.8	2.9b	1.9b	0.4b	0.1b	208b	46.5a	6.9b	0.8b
High metal – pH 6.8	3.1b	1.6c	0.1cd	0.1b	208b	27.1b	0.8d	0.2b
Low metal – pH 5.8	1.3c	0.9d	0.1d	0c	174c	19.9c	3.9c	0.8b
Low metal – pH 6.8	1.2c	0.8d	0.2c	0c	167c	10.7d	0d	0.2b

Note: Means followed by the same letter within the same column are not significantly different at  $P < 0.05$ .

**Table 3.** Shoot dry weight and metal concentration of *Thlaspi caerulescens* and *Trifolium pratense* after 55 days of growth.

Soil	Dry weight (g)	Shoot metal concn. (mg·kg <sup>-1</sup> )	
		Cd	Zn
<b><i>T. caerulescens</i></b>			
High metal – pH 5.8	0.189a	79.6a	2510b
High metal – pH 6.8	0.256a	22.4b	2780b
Low metal – pH 5.8	0.153a	18.8b	3370ab
Low metal – pH 6.8	0.194a	8.1c	6240a
<b><i>T. pratense</i></b>			
High metal – pH 5.8	1.084b	15.3a	281.9b
High metal – pH 6.8	1.511a	5.5b	138.5d
Low metal – pH 5.8	1.076b	3.3c	409.6a
Low metal – pH 6.8	1.722a	1.9d	158.6c

Note: Means followed by the same letter within the same column are not significantly different at  $P < 0.05$ .

**Table 4.** Effects of soil, *Thlaspi caerulescens*, and *Trifolium pratense* on culturable bacteria (Btot), culturable fungi (Ftot), numbers of Zn-resistant bacteria (Zn-R) and Cd-resistant bacteria (Cd-R), and the proportions of fungi (F/B), Zn-resistant bacteria (Zn-R/B), and Cd-resistant populations (Cd-R/B) compared with culturable bacteria after 95 days growth.

Soil	Microbial numerations (CFU/g (wet rhizosphere))				Proportion of subpopulations		
	Btot	Ftot	Zn-R	Cd-R	F/B	Zn-R/B	Cd-R/B
High metal – pH 5.8							
Bulk	2.76×10 <sup>7</sup> b	5.27×10 <sup>4</sup> c	4.63×10 <sup>5</sup> c	3.88×10 <sup>5</sup> c	1.91×10 <sup>-3</sup> c	1.68×10 <sup>-2</sup> b	1.40×10 <sup>-2</sup> b
Rhizosphere							
<i>T. pratense</i>	3.87×10 <sup>8</sup> a	2.93×10 <sup>6</sup> a	2.79×10 <sup>7</sup> a	2.57×10 <sup>7</sup> a	6.83×10 <sup>-3</sup> b	5.15×10 <sup>-2</sup> ab	5.68×10 <sup>-2</sup> a
<i>T. caerulescens</i>	2.67×10 <sup>7</sup> b	3.90×10 <sup>5</sup> b	2.29×10 <sup>6</sup> b	2.07×10 <sup>6</sup> b	1.46×10 <sup>-2</sup> a	8.58×10 <sup>-2</sup> a	7.82×10 <sup>-2</sup> a
High metal – pH 6.8							
Bulk	4.62×10 <sup>7</sup> c	6.69×10 <sup>4</sup> c	1.48×10 <sup>5</sup> c	1.10×10 <sup>5</sup> c	1.45×10 <sup>-3</sup> b	3.20×10 <sup>-3</sup> b	2.38×10 <sup>-3</sup> b
Rhizosphere							
<i>T. pratense</i>	5.71×10 <sup>8</sup> a	2.86×10 <sup>6</sup> a	3.29×10 <sup>7</sup> a	1.43×10 <sup>7</sup> a	5.00×10 <sup>-3</sup> a	5.77×10 <sup>-2</sup> a	2.51×10 <sup>-2</sup> a
<i>T. caerulescens</i>	1.20×10 <sup>8</sup> b	6.28×10 <sup>5</sup> b	2.98×10 <sup>6</sup> b	2.61×10 <sup>6</sup> b	5.23×10 <sup>-3</sup> a	2.48×10 <sup>-2</sup> a	2.18×10 <sup>-2</sup> a
Low metal – pH 5.8							
Bulk	3.01×10 <sup>7</sup> c	1.46×10 <sup>5</sup> b	3.16×10 <sup>5</sup> b	1.79×10 <sup>5</sup> b	4.87×10 <sup>-3</sup> b	1.05×10 <sup>-2</sup> b	5.95×10 <sup>-3</sup> b
Rhizosphere							
<i>T. pratense</i>	3.06×10 <sup>8</sup> a	5.65×10 <sup>5</sup> a	5.17×10 <sup>6</sup> a	4.22×10 <sup>6</sup> a	1.84×10 <sup>-3</sup> c	1.69×10 <sup>-2</sup> b	1.38×10 <sup>-2</sup> b
<i>T. caerulescens</i>	5.24×10 <sup>7</sup> b	7.11×10 <sup>5</sup> a	8.36×10 <sup>6</sup> a	3.94×10 <sup>6</sup> a	1.36×10 <sup>-2</sup> a	1.60×10 <sup>-1</sup> a	7.53×10 <sup>-2</sup> a
Low metal – pH 6.8							
Bulk	3.03×10 <sup>7</sup> b	3.60×10 <sup>4</sup> b	6.35×10 <sup>5</sup> b	2.18×10 <sup>5</sup> b	1.13×10 <sup>-3</sup> b	2.10×10 <sup>-2</sup> b	7.20×10 <sup>-3</sup> b
Rhizosphere							
<i>T. pratense</i>	1.99×10 <sup>8</sup> a	3.91×10 <sup>5</sup> a	5.07×10 <sup>6</sup> a	1.92×10 <sup>6</sup> a	1.96×10 <sup>-3</sup> b	2.54×10 <sup>-2</sup> b	9.62×10 <sup>-3</sup> b
<i>T. caerulescens</i>	4.47×10 <sup>7</sup> a	2.64×10 <sup>5</sup> a	8.11×10 <sup>6</sup> a	3.77×10 <sup>6</sup> a	4.78×10 <sup>-3</sup> a	2.10×10 <sup>-1</sup> a	8.43×10 <sup>-2</sup> a

Note: Means followed by the same letter within the same column are not significantly different at  $P < 0.05$ .

1994, 1995) (Table 2). Liming of soil is known to decrease metal availability by precipitation and chelation on new binding sites produced by the deprotonation of some functional groups (Sparks 1995).

The decrease in metal toxicity increased growth of both plants in the high-metal soil (Table 3). After liming, *T. caerulea* grew well on all soil-pH combinations. While both Zn and Cd shoot concentrations were elevated as expected for a hyperaccumulator, levels did not approach toxicity for either species (Brown et al. 1994, 1995). In fact, Zn shoot concentrations were higher for the low-metal soil, showing the ability of this species to "scavenge" metals from soil. Inexplicably, Zn uptake by *T. caerulea* was not pH dependent. If true, this observation may have a significant influence on practical phytoremediation, in that pH may not need to be reduced as much as originally thought to maintain metal uptake over multiple croppings. *Trifolium pratense* exhibited slight toxicity and a reduced growth at the low pH of 5.8, which was correlated with high-metal shoot content approaching phytotoxic levels for a nonhyperaccumulator plant.

The analysis of microbial populations in the rhizosphere demonstrated that microbes were affected by the presence of each plant (Table 4). Higher numbers of bacteria, fungi, and Cd-resistant bacteria and Zn-resistant bacteria were found in the rhizosphere of both *T. caerulea* and *T. pratense* compared with the bulk soil. Higher microbial populations in the rhizosphere are commonly described as the result of improvement in soil conditions related to greater quantities of organic compounds and surfaces for microbial colonization (Lynch and Whipps 1990; Grayston et al. 1997; Westover et al. 1997; Tate 2000). Higher numbers of culturable bacteria and fungi were observed in the rhizosphere of *T. pratense* compared with *T. caerulea*. It is known that plant effects on microbes vary greatly with the nature and amount of organic compounds released by the roots (Bowen and Rovira 1991), and that rhizosphere microbes isolated from different plants utilize different carbon sources (Grayston et al. 1997). The slow-growing nature of *T. caerulea* may result in a lower quantity of exudates and a reduced impact upon the rhizosphere compared with *T. pratense*.

Higher proportions of fungi and Cd-resistant bacteria and Zn-resistant bacteria, compared with the total bacterial population, were found in the rhizosphere of *T. caerulea* compared with the bulk soil and with the rhizosphere of *T. pratense*, with the exception of the high-metal soil – pH 6.8 combination. Since metal availability and toxicity in soil are related to soil pH (Sanders 1983), a decrease in the rhizosphere pH by *T. caerulea* is hypothesized to explain the higher proportion of metal-resistant bacteria and fungi found in the rhizosphere of *T. caerulea* compared with *T. pratense*. This hypothesis is supported by McGrath et al. (1997), who observed that the rhizosphere of *T. caerulea* has a lower pH (0.2–0.4) than nonrhizosphere soil.

In summary, the presence of *T. caerulea* or *T. pratense* enhanced microbial populations in the metal-contaminated soil. However, the two plants differed in their effect on rhizosphere microbes. *Trifolium pratense* supported higher numbers of bacteria and fungi, and *T. caerulea* exhibited

a higher proportion of metal-resistant bacteria and fungi within the total microbial population. This may be the result of a lower rhizosphere pH associated with the plant's ability to decrease soil pH to increase the availability and uptake of the metals.

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