Influence of root activity on speciation and solubility of nutrients and metals in the rhizosphere

Author(s): Dessureault-Rompre, Jacynthe

Publication Date: 2007

Permanent Link: https://doi.org/10.3929/ethz-a-005507963

Rights / License: In Copyright - Non-Commercial Use Permitted
Influence of root activity on speciation and solubility of nutrients and metals in the rhizosphere

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
For the degree of
Doctor of Sciences

Presented by
JACYNTHE DESSUREAULT-ROMPRE
MSc Soil and Environmental Science, Laval University
Born 14th March 1977
Citizen of Canada

Accepted on the recommendation of
Prof. Rainer Schulin, examiner
Prof. Emmanuel Frossard, co-examiner
PD Dr. Bernd Nowack, co-examiner
Dr. Jörg Luster, co-examiner

2007
### Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>III</td>
</tr>
<tr>
<td>RESUME</td>
<td>VI</td>
</tr>
<tr>
<td><strong>1 INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 Objectives of this study</td>
<td>3</td>
</tr>
<tr>
<td>1.2 References</td>
<td>5</td>
</tr>
<tr>
<td><strong>2 MODIFIED MICRO SUCTION CUP/RHIZOBOX APPROACH FOR THE IN-SITU DETECTION OF ORGANIC ACIDS IN RHIZOSPHERE SOIL SOLUTION</strong></td>
<td>7</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>8</td>
</tr>
<tr>
<td>2.2 Material and Methods</td>
<td>9</td>
</tr>
<tr>
<td>2.2.1 Rhizobox</td>
<td>9</td>
</tr>
<tr>
<td>2.2.2 Soil solution sampling</td>
<td>10</td>
</tr>
<tr>
<td>2.2.3 Soil solution analysis</td>
<td>12</td>
</tr>
<tr>
<td>2.3 Results</td>
<td>13</td>
</tr>
<tr>
<td>2.3.1 Sampling volume</td>
<td>13</td>
</tr>
<tr>
<td>2.3.2 Temporal variations of citrate in the rhizosphere of cluster roots</td>
<td>15</td>
</tr>
<tr>
<td>2.3.3 Comparison between rhizosphere and bulk soil solution</td>
<td>16</td>
</tr>
<tr>
<td>2.4 Discussion and conclusion</td>
<td>18</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>20</td>
</tr>
<tr>
<td>2.5 References</td>
<td>21</td>
</tr>
<tr>
<td><strong>3 SPATIAL AND TEMPORAL VARIATION IN ORGANIC ACID ANION EXUDATION AND NUTRIENT ANION UPTAKE IN THE RHIZOSPHERE OF LUPINUS ALBUS L.</strong></td>
<td>23</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>24</td>
</tr>
<tr>
<td>3.2 Material and Methods</td>
<td>26</td>
</tr>
<tr>
<td>3.2.1 Rhizobox system</td>
<td>26</td>
</tr>
<tr>
<td>3.2.2 Soil solution sampling</td>
<td>27</td>
</tr>
<tr>
<td>3.2.3 Soil solution analysis</td>
<td>28</td>
</tr>
<tr>
<td>3.3 Results</td>
<td>29</td>
</tr>
<tr>
<td>3.3.1 Comparison between bulk and rhizosphere soil solutions</td>
<td>29</td>
</tr>
<tr>
<td>3.3.2 Temporal variability of organic acid anions in the cluster root rhizosphere</td>
<td>30</td>
</tr>
<tr>
<td>3.3.3 Temporal variability of inorganic anions in the cluster root rhizosphere</td>
<td>32</td>
</tr>
<tr>
<td>3.3.4 Spatial distribution of organic acid and inorganic anions in the rhizosphere of cluster roots</td>
<td>33</td>
</tr>
<tr>
<td>3.3.5 Mobilization of phosphate by citrate in batch experiments</td>
<td>35</td>
</tr>
<tr>
<td>3.4 Discussion</td>
<td>35</td>
</tr>
<tr>
<td>3.4.1 Influence of sampling and system fluxes</td>
<td>35</td>
</tr>
<tr>
<td>3.4.2 Root exudation by cluster roots</td>
<td>36</td>
</tr>
<tr>
<td>3.4.3 Root exudation and phosphate concentration in the rhizosphere soil solution of cluster roots</td>
<td>38</td>
</tr>
<tr>
<td>3.4.4 Exudation and nutrient uptake by normal roots and nodules</td>
<td>39</td>
</tr>
<tr>
<td>3.5 Conclusions</td>
<td>40</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>41</td>
</tr>
<tr>
<td>3.6 References</td>
<td>42</td>
</tr>
<tr>
<td><strong>4 METALSOLUBILITY AND SPECIATION IN THE RHIZOSPHERE OF LUPINUS ALBUS L.</strong></td>
<td>45</td>
</tr>
<tr>
<td>4.1 Introduction</td>
<td>45</td>
</tr>
<tr>
<td>4.2 Materials and Methods</td>
<td>48</td>
</tr>
<tr>
<td>4.2.1 Rhizobox system</td>
<td>48</td>
</tr>
<tr>
<td>4.2.2 Soil solution sampling</td>
<td>48</td>
</tr>
</tbody>
</table>
5 MOBILIZATION AND COMPLEXATION OF ZN AND Cd IN THE RHIZOSPHERE OF THLASPI CAERULESCENS

ABSTRACT .......................................................................................................................... 67

5.1 INTRODUCTION ........................................................................................................ 68

5.2 MATERIAL AND METHODS .................................................................................. 70

5.2.1 Rhizobox system .................................................................................................. 70

5.2.2 Soil solution sampling ......................................................................................... 71

5.2.3 Soil solution analysis ........................................................................................... 72

5.2.4 Complexation studies based on the dissociation of metal complexes .............. 73

5.2.5 Speciation calculations ......................................................................................... 74

5.2.6 Plant metal concentrations .................................................................................. 74

5.3 RESULTS .................................................................................................................. 74

5.3.1 Metal concentrations in roots and shoots ............................................................. 74

5.3.2 pH, inorganic anions, DOC, oxalate concentrations and specific absorbance in rhizosphere and plant-free soil ................................................................. 77

5.3.3 Total metal concentrations in solutions ............................................................... 77

5.3.4 Time evolution of total and % dynamic Cu, Zn, Cd and Pb in the rhizosphere and plant-free soil solutions ................................................................................. 78

5.3.5 pH dependence of the dissociation of the metal complexes ............................ 79

5.4 DISCUSSION .......................................................................................................... 81

5.4.1 Metal concentrations in roots and shoots ............................................................. 81

5.4.2 Total metal concentrations .................................................................................. 81

5.4.3 Metal speciation .................................................................................................. 82

5.4.4 Origin of the complexing ligands in the rhizosphere ........................................ 83

ACKNOWLEDGEMENTS ................................................................................................. 84

6 CONCLUSIONS ............................................................................................................. 89

6.1 MODIFIED MICRO SUCTION CUP/ RHIZOBOX APPROACH FOR THE IN-SITU DETECTION OF ORGANIC ACIDS IN RHIZOSPHERE SOIL SOLUTION ................................................................................................................................. 89

6.2 ORGANIC ACID EXUDATION AND NUTRIENT ANIONS IN THE RHIZOSPHERE SOIL SOLUTION OF LUPINUS ALBUS ................................................................................................................................. 90

6.3 METAL SOLUBILITY AND SPECIATION IN THE RHIZOSPHERE OF LUPINUS ALBUS ................................................................................................................................. 90

6.4 MOBILIZATION AND COMPLEXATION OF ZN AND Cd IN THE RHIZOSPHERE OF THLASPI CAERULESCENS ................................................................................................................................. 91

6.5 OUTLOOK AND OPEN QUESTIONS ......................................................................... 91

ACKNOWLEDGEMENTS ................................................................................................. 93

CURRICULUM VITAE ........................................................................................................ 94
Abstract

Root-soil interactions can strongly influence the soil solution chemistry in the rhizosphere. Understanding the rhizosphere influence on the solubility and mobility of metals and heavy metals in soil solution may help improving soil remediation technique such as phytoremediation, a green and low-cost developing technology that can potentially address the problems of contaminated land. In the present study we first investigated a modification of the classical rhizobox/micro suction cup system to make it suitable for the collection and analysis of organic anions in the rhizosphere. In order to show the potential of the method, we tested the modified system with *Lupinus albus* as a model plant known to exude large amounts of citrate. The suction cups were installed through the transparent front plate of the rhizoboxes just after the emergence of cluster roots in order to allow optimal localized collection of soil solution. A small dead-volume allowed almost immediate stabilization with formaldehyde of the sampled soil solutions in the collection container to prevent microbial degradation. The concentrations of organic anions were significantly larger in the rhizosphere soil solution of active cluster roots of *Lupinus albus* than in the bulk soil solution. We were able to follow the exudation process which occurred during 2-3 days *in-situ*. The concentrations of other organic and inorganic anions differed between the bulk soil and the rhizosphere of cluster roots, normal roots and nodules.

In a second step we investigated *in-situ* the temporal patterns and spatial extent of organic acid anion exudation into the rhizosphere solution of *Lupinus albus*, and its relation with the nutrient anions phosphate, nitrate and sulfate by means of a rhizobox micro suction cup method under P sufficient conditions. We compared the soil solution in the rhizosphere of cluster roots with that in the vicinity of normal roots, nodules and bulk soil. Compared to the other rhizosphere and soil compartments, concentrations of organic acid anions were higher in the vicinity of cluster roots during the exudative burst (citrate, oxalate) and nodules (acetate, malate), while concentrations of inorganic nutrient anions were highest in the bulk soil. Both active cluster roots and nodules were most efficient in taking up nitrate and phosphate. The intensity of citrate exudation by cluster roots was highly variable. The overall temporal patterns during the lifetime of cluster roots were overlaid by a diurnal pattern, i.e. in most cases, the exudation burst consisted of one or more peaks occurring in the afternoon. Multiple exudation peaks occurred daily or were separated by
Abstract

one or two days. Although citrate concentrations decreased with distance from the cluster root apex, they were still significantly higher at a distance of 6 to 10 mm than in the bulk soil. Phosphate concentrations were extremely variable in the proximity of cluster roots. While our results indicate that under P sufficient conditions cluster roots take up phosphate during their entire life time, the influence of citrate exudation on phosphate mobilization from soil could not be assessed conclusively because of the complex interactions between P uptake, organic acid anion exudation and P mobilization. However, we observed indications of P mobilization concurrent with the highest measured citrate concentrations. In conclusion, this study provides semiquantitative in-situ data on the reactivity of different root segments of Lupinus albus L. in terms of root exudation and nutrient uptake under nutrient sufficient conditions, in particular on the temporal variability during the lifetime of cluster roots.

In a third step we investigated in-situ the influence of root exudation on nutrient and metal species in the rhizosphere of Lupinus albus using micro-suction cups. We found that large amounts of metals were mobilized in the rhizosphere of cluster roots during exudative bursts of citrate. DOC_{UV} concentrations (dissolved organic carbon without organic acid anions) increased in parallel with organic acid anion concentrations. Speciation calculations revealed that Ca, Mn, Al and Zn were mainly complexed by citrate. The speciation of Cu and Pb was not affected by citrate and they were strongly complexed by DOC. Citrate complexed metals in the order Ca > Al > Fe > Mg > Mn. The investigations show that the effect of citrate exudation on metal solubility was twofold: On one hand metals like Zn, Fe and Al were directly mobilized and complexed by citrate and on the other hand citrate was mobilizing DOC_{UV} which in turn then complexed and mobilized Cu and Pb.

In a fourth and last step, we investigated the mobilization and complexation of Zn and Cd in the rhizosphere of different Thlaspi species and ecotype, and their change over time. Our hypotheses were that Thlaspi caerulescens can release root exudates in its rhizosphere to enhance the mobilization and complexation of Zn and Cd and that there is difference between different ecotype and species. Using a modified rhizobox system, we found that the total Zn concentration in solution slightly increased with time in the rhizosphere of the ecotypes Thlaspi caerulescens Prayon and Gange. These results agree with the possibility of a mobilization of Zn and Cd in the rhizosphere solution of Thlaspi caerulescens. The dynamic fraction of Zn and Cd decreased considerably over time in the rhizosphere solution of Gange and
Prayon (only Zn), and were at the end of the experiment about 30 to 60 % less compared to *Thlaspi perfoliatum* and the plant-free soil. These results indicate that it is not primarily mobilization that is involved in the hyperaccumulation mechanism of Zn and Cd by *Thlaspi caerulescens* but that the complexation of those metals is influenced by the plants. The potential ligands in the rhizosphere of Ganges and Prayon are selective for Cd and Zn and do not change Cu and Pb speciation. UV absorbance spectroscopy results revealed that the rhizosphere solutions of *Thlaspi caerulescens* Gange and Prayon were characterized by higher specific absorbance compared to *Thlaspi perfoliatum* and the plant-free soil.
Résumé

Les interactions entre les racines et le sol peuvent fortement influencer la chimie de la solution du sol dans la rhizosphere. Mieux comprendre l’influence de la rhizosphere sur la solubilité et la mobilité des métaux et métaux lourds dans la solution du sol pourra aider à améliorer les techniques de remédiation du sol tel que la phytoREMÉDIATION. Dans la présente étude nous avons premièrement testé une modification de la version classique du système « rhizobox/micro suction cup » de façon à l’améliorer en vue de la collection et de l’analyse des anions organiques dans la solution de la rhizosphere. Dans le but de démontrer le potentiel de la méthode nous avons testé le système modifié avec Lupinus albus, une plante modèle qui est reconnue pour exuder de larges quantités de citrate. Les suction cups ont été installés à travers la fenêtre transparente avant du rhizoboxe, suivant l’émergence des racines protéoïques, de façon à permettre une collection localisée optimale de la solution du sol. Un petit « dead-volume » et l’ajout de formaldéhyde dans le contenant d’échantillonnage a permis une rapide stabilisation des échantillons prévenant ainsi la dégradation biologique. La concentration des anions organiques était significativement plus grande dans la solution de la rhizosphere près des racines protéoïques actives de Lupinus albus comparé à la solution non-rhizosphérique. Nous avons été capable de suivre in-situ le processus d’exudation qui est survenu pendant 2-3 jours. Des différences ont été observées, quant à la concentration des autres anions organiques et inorganiques entre les solutions des différentes rhizosphères (racines protéoïques, autres racines et nodules) et de la solution non-rhizosphérique.

Dans une seconde étape de cette étude, nous avons examiné in-situ l’évolution spatio-temporelle de l’exudation des anions organiques dans la rhizosphere de Lupinus albus et sa relation avec les nutriments anioniques comme le phosphate, le nitrate et le sulfate au moyen de la méthode modifiée du rhizoboxe. Nous avons comparé entre-elles les solutions des différentes rhizosphères ("cluster roots", autres racines, nodules) et de la solution non-rhizosphérique. La concentration des anions organiques était plus élevée à proximité des racines protéoïques pendant l’exudation maximale (citrate, oxalate), et des nodules (acetate, malate), alors que les concentrations en nitrate et en phosphate étaient plus élevées dans la solution non-rhizosphérique. Les racines protéoïques actives et les nodules étaient également efficaces dans
VII Résumé

l’assimilation du nitrate. Par contre, l’assimilation du phosphate a été plus intense au niveau des racines protéoïques. De plus, les nodules ont été un puit particulièrement fort pour le sulfate. La plus grande concentration en anions organiques dans la solution du sol près des racines protéoïques a été mesurée dans les deuxième partie de la journée (14 - 22h). En s’éloignant de la source de l’exudation, la concentration en anions organiques a diminué, mais est demeurée significativement plus élevée à une distance de 6 à 10 mm comparativement à la solution non-rhizosphérique. Les concentrations en nitrate et en phosphate dans la rhizosphere des racines protéoïques ont exhibé une variation diurnale marquée par une concentration plus élevée pendant la nuit soutenant le fait établi que les plantes assimilent moins d’élément nutritifs durant la période nocturne. L’intensité et la durée de l’exudation maximale des anions organiques ont varié entre les différents racines protéoïques. De plus, cette exudation est survenue en une ou plusieurs vagues (ou pulsations). Les concentrations de phosphate furent extrêmement variables à proximité des racines protéoïques. L’influence du citrate sur la mobilisation du phosphate n’a donc pas pu être clairement détectée étant donné les interactions complexes entre l’assimilation du phosphate, l’exudation des anions organiques et les phénomènes d’adsorption/désorption de cet anion ayant lieu entre la matrice du sol et la solution du sol.

Dans une troisième étape nous avons examiné in-situ l’influence de l’exudation racinaire sur les nutriments et les espèces métalliques dans la rhizosphere de *Lupinus albus*. Nous avons observé qu’une large quantité de cations métalliques était mobilisée dans la rhizosphere des racines protéoïques durant l’exudation maximale du citrate. Le contenu en carbone organique dissout (COD, sans tenir compte des anions organiques) a aussi augmenté avec l’augmentation de la concentration des anions organiques dans la solution de la rhizosphere. Les calculs de spéciation avec ECOSAT ont révélé que la présence des anions organiques ( principalement citrate) et l’augmentation du COD a eu un fort impact sur la complexation des métaux. Pour le Ca, le Mn et l’Al, le citrate fut un complexant plus important dans la solution de la rhizosphere comparé au COD. Pour le Fe et le Zn, la complexation avec le COD a diminué avec l’augmentation de la présence du citrate dans la solution de la rhizosphere. Cependant le COD était plus important pour la complexation la plupart du temps. Le Cu et le Pb étaient fortement complexé au COD. Le citrate était fortement complexé par les métaux dans l’ordre suivant : Ca > Al > Fe > Mg > Mn. Les résultats démontrent que l’effet du citrate sur la solubilité des métaux est à double effets : d’une part les métaux comme le Zn, le Fe, et l’Al sont directement mobilisés
et complexés par le citrate et de l’autre côté le citrate mobilise le COD qui en retour peut complexer et mobiliser le Cu et le Pb.

Le quatrième et dernier objectif de cette étude était d’examiner la mobilisation et la complexation du Zn et du Cd dans la rhizosphère de différentes espèces de *Thlaspi* et ses changement dans le temps (avec le développement racinaire). Nous avons observé que le développement ou l’activité racinaire plus intense n’engendrait pas une importante modification de la concentration totale des métaux en solution. Cependant, la concentration totale du Zn dans la solution de la rhizosphère pour les populations de *Thlaspi* Gange et Prayon a augmenté légèrement dans le temps. Ces résultats soutiennent la possibilité de la mobilisation du Zn et du Cd dans la rhizosphère de *Thlaspi caerulescens* comme mécanisme de l’hyperaccumulation. Par contre, la fraction dynamique du Zn et du Cd a diminué considérablement dans le temps dans la solution de la rhizosphère de *Thlaspi* Gange et Prayon et nous avons observé une importante et significative différence comparé aux résultats obtenus pour *Thlaspi Perfoliatum* et pour la solution non-rhizosphérique à la fin de l’expérience. Ces résultats pourraient indiquer que ce n’est pas principalement la mobilisation qui est impliquée dans le mécanisme d’hyperaccumulation du Zn et du Cd par *Thlaspi caerulescens* mais plus particulièrement que la complexation des ces métaux est influencée par ces plantes. Les ligands potentiels dans la rhizosphère de *Thlaspi* Gange et Prayon sembleraient être sélectifs pour le Cd et le Zn puisqu’ils ne changent pas la spéciation du Cu et du Pb. La spectroscopie d’absorbance UV a révélée que la solution de la rhizosphère de *Thlaspi caerulescens* Gange et Prayon était caractérisée par une absorbance spécifique plus grande comparé à celle de *Thlaspi perfoliatum* et de la solution non-rhizosphérique. Le COD des ces deux populations d’hyperaccumulateurs semble donc caractérisé par des propriété aromatiques et hydrophobiques plus importantes qui pourraient jouer un rôle majeur dans les complexation du Zn et du Cd et de ce fait dans le mécanisme d’hyperaccumulation par *Thlaspi caerulescens*.
1 Introduction

Ongoing and past contamination of soils by heavy metals remains a serious problem in an increasing number of areas. The build up of metals such as Cu, Zn, Cd and Pb in soils can lead to toxicity for soil microbes, soil fauna, higher animals, plants and humans (McGrath et al. 2002). This metallic contamination in soils originates from many processes including atmospheric deposition, industrial activities, disposal of wastes such as sewage sludge, animal manure, ash, domestic and industrial wastes, and from the utilization of fertilizers, lime or agrochemicals.

Environmental hazards originating from heavy metals are closely related to their solubility and mobility in soil profiles. Solubility and mobility of heavy metals in soils are controlled by many parameters such as soil properties including water regime, mineralogy, organic matter content and one of the main concerns of this work, the presence of plants and the associated rhizosphere.

The demand for remediation techniques to clean-up heavy metals contaminated soils is increasing continuously. No low-cost technique is currently available to clean up moderately contaminated soils and to ensure that soils retain their fertility after metal removal. Phytoremediation is a developing technology that can potentially address the problems of contaminated agricultural land or more intensely polluted areas affected by urban or industrial activities. Three main strategies currently exist to phytoextract inorganic substances from soils using plants (McGrath et al. 2002): 1) the use of natural hyperaccumulators; 2) the enhancement of element uptake by high biomass species through chemical additions to soil and plants; 3) the phytovolatization of volatile elements. This work is focusing on the first strategy.

Hyperaccumulators are plants exhibiting an extraordinary capacity to concentrate heavy metals in their shoots (Baker and Brooks 1989). Metal hyperaccumulation is a rare phenomenon in terrestrial higher plants and has been found for Ni (Baker et al. 2000), Zn and Cd (Baker et al. 2000), Cu and Co (Brooks 1998; Reeves and Baker 2000), Se and As (Brooks 1998; Ma et al. 2001; Reeves and Baker 2000).

The interface between plants and the soil is the rhizosphere. The term rhizosphere from Greek, meaning the influence of root on its surrounding was first used by Hiltner, (1904) to indicate the zone of soil where root exudates released from plant roots can stimulate inhibit or have no effect on activities of soil microorganisms. Today the term rhizosphere has a more general concept and
is defined as the volume of soil around living plant roots that is influenced by root activities (Darrah 1991; Hinsinger 1998).

The conditions in the rhizosphere differ in many respects from those in the soil at some distance from the root, the so-called bulk soil. Roots act as a sink for mineral nutrients transported to the root surface by mass flow and diffusion. In addition they take up either ions or water preferentially which may lead to the depletion or accumulation of ions. They also release $H^+$ or $HCO_3^-$ (and $CO_2$) which changes the pH, and they consume or release $O_2$ which may cause alterations in the redox potential (Marschner 1995).

A multitude of other compounds are released into the rhizosphere, most of which are organic compounds and are normal plant constituents derived from photosynthesis and other plant processes. The amount of these compounds produced by plant roots varies with plant species, cultivar, the age of plant, the environmental conditions such as soil properties and the level of physical, chemical and biological stress (Pinton et al. 2001). Root exudates comprise both high and low-molecular weight solutes. The most important components of the high-molecular weight solutes are mucilage and ectoenzymes and of the low-molecular weight fractions organic acids, sugars, phenolics and amino acids including phytosiderophores. Low-molecular weight organic acids (e.g. citrate) may influence the solubility of mineral nutrients as well as heavy metals directly or indirectly by providing energy for microbial activities in the rhizosphere (Jones and Darrah 1994). Due to an exceptionally high exudation rate of citrate in case of phosphate deficiency, *Lupinus albus* is one of the best model plants for understanding the exudation phenomenon in relation to nutrient deficiency.

The effect of root exudation on DOC concentration in rhizosphere solution hasn’t received much attention so far. DOC plays an important role in the biochemistry of carbon, nutrients and pollutants in soils because of chelation with polyvalent cations, cation exchange and metal solubility (Stevenson 1994). Organic anions can increase soil derived DOC indirectly through an increase of the microbial activity and increased mobilization of soil organic matter by the microorganisms. High concentrations of organic anions are known to directly mobilize DOC from soils, presumably through complexation of Ca that is stabilizing the organic matter in the soil (Yang et al. 2001; Hauser et al. 2005).

Understanding how plants accumulate and store metal ions is relevant on behalf of human nutrition agriculture, and metal detoxification using plants as biological detoxification systems
for the phytoremediation of metal contamination in the environment. Metals ions such as Cu, Fe, Zn and Se are essential nutrients for which deficiencies in animal and human diets cause significant disorders. Cd, Hg, Pb, Al and As non-essential metals can cause toxicities when present in excess. Knowledge about the mechanisms by which both essential and nonessential metals can be sequestered, stored and detoxified may contribute to the optimization of phytoremediation processes (Clemens et al. 2002). Processes occurring in the rhizosphere of hyperaccumulator plants like the well known Thlaspi caerulescens have been studied extensively. Nevertheless the mechanism of metal hyperaccumulation is still poorly understood.

1.1 Objectives of this study

One of the main concerns of this work was to get a better understanding of the rhizosphere processes leading to hyperaccumulation of metals by plants. To reach this major objective, we first had to deal with one of the main issues in rhizosphere research: the in situ sampling of the rhizosphere solution.

The use of micro-techniques for the collection of soil solution enables non-destructive in-situ observation of soil solution chemistry at high spatial and temporal resolution (Grossmann and Udluft 1991). By contrast, obtaining the soil solution by soil sampling followed by centrifugation or by extraction with water or salt solution is operationally strongly biased, and, because sampling is destructive, does not allow for time studies (Wolt 1994). Soil solution investigations at the microscale started in the 1990s with the miniaturization of the sampling probes (Göttlein et al. 1996; Vetterlein and Marschner 1993; Vetterlein et al. 1993). Later, micro suction cups were used in conjunction with rhizotrones, also called rhizoboxes, that allow observing the development of root systems through a transparent front plate (Dieffenbach et al. 1997). This approach allowed for the first time to monitor the soil solution chemistry with high spatial resolution for rhizosphere studies without destroying the soil structure. However, there are only a few in-situ studies of organic anions in solution and their impact on metals solubility as well as few in-situ studies that are looking deeply in the rhizosphere solution processes involved in the hyperaccumulator plants.
With this in mind the objectives of the present work were:

i. To test a modification of the classical rhizobox micro suction cup system design to obtain a better precision of the localized in-situ sampling of soil solution and, in addition, to minimize the risk of biodegradation in the samples (Chapter 2).

ii. To investigate in-situ the temporal and spatial patterns of organic anions exuded by cluster roots of *Lupinus albus* and their relation to soil solution chemistry, in particular to nutrients such as phosphate, nitrate and sulfate (Chapter 3).

iii. To investigate the impact of the exudation of organic anions by *Lupinus albus* on the concentration of macro and micronutrients in different rhizosphere and soil solution samples. In addition we performed speciation measurements and calculations to reveal changes in metal speciation during exudation (Chapter 4).

iv. To investigate metal solubility and complexation in the rhizosphere solution of *Thlaspi caerulescens* compared to the non-hyperaccumulator *Thlaspi perfoliatum* and the bulk soil (Chapter 5).
1.2 References


Brooks R R 1998 Plants that hyperaccumulate heavy metals, Wallingford UK.


Chapter 1 Introduction


2 Modified micro suction cup/ rhizobox approach for the \textit{in-situ} detection of organic acids in rhizosphere soil solution

Jacynthe Dessureault-Rompré, Bernd Nowack, Rainer Schulin, Jörg Luster


Abstract

Root-soil interactions can strongly influence the soil solution chemistry in the rhizosphere. In the present study we propose a modification of the classical rhizobox/micro suction cup system to make it suitable for the collection and analysis of organic acids in the rhizosphere. In order to show the potential of the method, we tested the modified system with \textit{Lupinus albus} L. as a model plant known to exude large amounts of citrate. The suction cups were installed through the transparent front plate of the rhizoboxes just after the emergence of cluster roots in order to allow optimal localized collection of soil solution. A small dead-volume allowed almost immediate stabilization with formaldehyde of the sampled soil solutions in the collection container to prevent microbial degradation. The concentrations of organic acids were significantly larger in the rhizosphere soil solution of active cluster roots of \textit{Lupinus albus} L. than in the bulk soil solution (about 400 \(\mu\)M of citrate vs. <0.05 \(\mu\)M). We were able to follow the exudation process which occurred during 2-3 days \textit{in-situ}. Also the concentrations of other organic acids and inorganic anions differed between the bulk soil and the rhizosphere of cluster roots, normal roots and nodules.
2.1 Introduction

Active plant roots have been recognized to influence soil solution chemistry in their vicinity (Marschner, 1995). This rhizosphere differs in many aspects from the bulk soil due to root activity and higher microbial and fungal activities. Root exudation of low-molecular weight organic acids has been found to play an important role in the mobilisation and uptake of nutrients as well as the detoxification of harmful substances (Jones, 1998; Marschner, 1995; Ryan et al., 2001). Most of our knowledge on the exudation of organic acids stems from studies in hydroponic systems (e.g., Neumann et al., 1999; Watt and Evans, 1999) or soil extracts (e.g., Dinkelaker et al., 1989; Gerke et al., 1994; Jones and Darrah, 1994; Li et al., 1997). However, roots in hydroponic systems can behave differently compared to natural conditions in soils (Marschner, 1995). Very little is known about the behavior of organic acids in soil solution in terms of mobility and degradability. Thus, there is a large need for in-situ studies in soils.

The use of micro-techniques for the collection of soil solution enables non-destructive in-situ observation of soil solution chemistry at high spatial and temporal resolution (Grossmann and Udluft, 1991). By contrast, obtaining the soil solution by soil sampling followed by centrifugation or by extraction with water or salt solution is operationally strongly biased, and, because sampling is destructive, does not allow for time studies (Wolt, 1994). Soil solution investigations at the microscale started in the 1990s with the miniaturization of the sampling probes (Göttlein et al., 1996; Vetterlein and Marschner, 1993; Vetterlein et al., 1993). Later, micro suction cups were used in conjunction with rhizotrones, also called rhizoboxes, that allow observing the development of root systems through a transparent front plate (Dieffenbach et al., 1997). This approach allowed for the first time to monitor the soil solution chemistry with high spatial resolution for rhizosphere studies without destroying the soil structure. This technique has been used to study aluminium and nutrient chemistry in the rhizosphere in the laboratory (Arocena et al., 2004; Göttlein et al., 1999; Wang et al., 2004) as well as in the field (Braun et al., 2001; Göttlein and Matzner, 1997; Hagedorn et al., 1999). However, there are only a few in-situ studies of organic acids in soil solution. Concentrations of organic acids in forest floor leachates collected by means of zero-tension lysimeters were found to be between 1 and 50 μM (Krzyszowska et al., 1996; Strobel, 2001). Sandnes et al. (2005) observed low concentrations of organic acids (<1 – 80 μM for di- and tricarboxylic acids) in a study comparing samples collected
from rhizoboxes and microcosms planted with birch and spruce with samples from root windows installed in the field in the root zone of these trees. The rhizobox study showed higher concentrations of organic acids which was related to a higher root and fungal density and activity. No comparison between rhizosphere and bulk soil solution and no stabilisation of the samples was done in this study.

The objective of this study was to test a modification of the classical rhizobox micro suction cup system design to obtain a better precision of the localized in-situ sampling of soil solution and, in addition, to minimize the risk of biodegradation in the sample. We chose *Lupinus albus* as a test plant because it is well known to exude high amounts of citrate into the rhizosphere by using special roots called cluster roots to cope with phosphate deficiency (Dinkelaker et al., 1995).

### 2.2 Material and Methods

#### 2.2.1 Rhizobox

The rhizobox used in this study was adapted from the one introduced by Dieffenbach et al. (1997). Each box had a length of 60 cm, a width of 15 cm and a depth of 1 cm in the main rooting compartment (inner volume around 900 cm$^3$). Only one seedling was planted per box. The front side of the rhizobox was covered with a transparent plate made from acrylic glass to allow visual observation of the development of the roots. The three other sides of the rhizobox were made from dark polyvinylchloride (PVC). The rhizobox was positioned at an angle of 30° to force the roots to grow along the transparent plate. The transparent side of the rhizobox was covered with a dark cardboard box to prevent light interference on root growth. The cardboard was removed only to observe the root development and to take samples. Seeds of *Lupinus albus* L. ("Weissblühende Tellerlupin" cultivar, Ufa AG, Switzerland) were pre-treated with 10% hydrogen peroxide (Liang and Li, 2003) and then germinated in black garden soil for one week. Healthy plants were gently washed with deionised water to remove the organic black soil transplanted into the rhizoboxes. We used a carbonate free soil (pH 6.4 (0.01 M CaCl$_2$), 15.1 g/kg C$_{org}$, 1.5 g/kg N$_{tot}$, 49 mg/kg P$_{available}$ (0.5 M NaHCO$_3$, pH 8.5, Kuo, 1996) (Kuo 1996), 862
mg/kg P<sub>org</sub> (Kuo, 1996), 36% sand, 49% silt, 15% clay). The soil was air dried, sieved (2 mm) and filled into the rhizoboxes at a bulk density of about 1.2 g cm<sup>-3</sup>.

The rhizobox experiment was conducted under controlled conditions in a climate chamber (light 16 h per day with an intensity at canopy height of 150 μm m<sup>-2</sup> s<sup>-1</sup>, 80% humidity, temperature day/night: 20/16°C). The boxes were irrigated with synthetic rain water (ionic composition in μM: 70 NH<sub>4</sub>, 70 NH<sub>3</sub>, 3.2 PO<sub>4</sub>, 17 Cl, 3.1 SO<sub>4</sub>, 4.3 Na, 7.7 K, 5 Ca, 1.3 Mg, 0.15 Zn, pH =5.5) using wicks that were made from a polymerous tube (Rhizon irrigators, Rhizosphere research products, Wageningen Netherlands) and installed at 5, 30 and 55 cm soil depth. A water potential of -40 hPa was applied by means of a hanging water column between the wicks and the reservoir.

**2.2.2 Soil solution sampling**

Samples were taken through the transparent front plate of the rhizoboxes and not from the back side (Figure 1.1) as in the original design by Dieffenbach et al. (1997). This modification was made because in the rhizobox root growth is not necessarily restricted to the soil close to the front plate. Some roots can also grow along the backside of the box despite the 30° inclination of the boxes. The conventional way of installing the micro suction cups through the back side of the rhizobox does not allow to determine with certainty whether a sample represents rhizosphere or bulk soil solution. Installation of the cups through the transparent front plate assures that the “active length” of the micro suction cup of 5 mm is near the root of interest. To optimise the precise positioning of the suction cups, holes were drilled through the front plate at a given location well defined in terms of distance to a root just before insertion of the cups by using a hand drill. In order to sample the rhizosphere soil solution around cluster roots, the emergence of the rootlets was the starting point of sampling. Each cluster root was sampled for a period of 7 to 10 days, once a day for a period of 8 hours. The sampling-free time of 16 hours accounts for the need of the soil to reequilibrate, because each time soil solution is sampled, water is removed from the sampling site and the conditions for water and nutrient chemistry are changed (Vetterlein and Jahn, 2004). Micro suction cups were positioned around the cluster root at three different distances to get spatial information. The three layers of micro suction cups (< 1 mm from the rootlet apex, 1-5 mm and 6-10 mm) were installed in such a way that the interaction between
the individual cups was minimised. One millilitre syringes (Norm-Ject, Henke Sass Wolf, Tuttlingen Germany) connected to the micro suction cups were used to collect the soil solution (Figure 1). Twenty µL of formaldehyde solution (> 36.5% (Total) in water (Fluka)) were added to each syringe before the sampling to prevent microbial modification of the collected samples. In order to verify the ability of formaldehyde to keep the soil solution composition constant we analysed different samples from 1 to 4 weeks after the first analysis. We found out that the concentrations of inorganic anions and organic acids is not changing over time, thus proving the efficiency of the formaldehyde. Vacuum was applied once by pulling the piston of the syringe to its end position. With the type of syringe used, the piston remained at this position for the whole sampling period without or little fixing using adhesive tape.

Ceramic capillaries (pure aluminium oxide produced by PI ceramic, D-07589 Lederhose Germany) were used as micro suction cup materials (Göttlein et al., 1996). Pieces of 1 cm length were connected to Tygon tubes (R3607 0.89 mm (ID), 0.86 mm (WALL), (OmniLab, Mettmenstetten Switzerland)) and to a female luer connector (Figure 1). A smaller PTFE tube (Tube 13902267 1/32 X 0.25mm (OmniLab, Mettmenstetten Switzerland)) was inserted into the Tygon tube to reduce the total dead volume to 4 µL. The tips of the capillaries were closed with common hot glue. Each micro suction cup was tested for leaks. Different to the usual conditioning, the micro suction cups were first sterilized with ethanol. Also, to minimise adsorption of metals onto the suction cup materials, they were conditioned by passing up to 10 mL of autoclaved soil solution obtained by a batch soil water extract (soil/water ratio 1/10). Adsorption of low molecular weight organic acids on these ceramic capillaries material was reported to be negligible (Sandnes et al., 2005).
2.2.3 Soil solution analysis

For each soil solution sample, the sampling volume was recorded. The samples were analyzed for low molecular weight organic acids (LMWOA) (citrate, malate, oxalate, formate, acetate) and inorganic anions (sulfate, nitrate, and phosphate) using ion chromatography (Dionex autosampler system, AS 50 column, eluent generator: potassium hydroxide (1 to 60 mM), flow: 1.5 mL min⁻¹) with 200 μL insert glass vials to reduce the sample volume. The pH was measured by an ion sensitive field effect transistor electrode (ISFET sensor, Sentron, Roden The Netherlands). For the cluster roots, the sampling period was divided into the three sub periods “before”, “during” and “after” LMWOA exudation. “During” was defined as the period during which the concentration of citrate was increased 10-times above the background. During the experiment, 5 cluster roots were sampled. The soil solution in the rhizosphere of normal roots (along long roots and near lateral roots) and nodules as well as in the bulk soil (> 2 cm from the nearest root) was also sampled. Statistical differences were tested using Student’s t-test (Systat 11). Pearson correlation coefficients between the sampling volume and concentrations of organic acids and inorganic anions were calculated using Systat 11.
Chapter 2 Modified micro suction cup/rhizobox approach

The formaldehyde added to prevent microbial degradation contains some impurities, mainly formate, acetate and chloride with traces of some cations (Al, Mn, and Pb). For a soil solution sample of 400 μL these impurities correspond to a concentration of 150 μM formate, 28 μM acetate, and 5.4 μM chloride. Formaldehyde solution can also affect the pH measurement at small sampling volumes. Titration curves with formaldehyde were measured on soil water extracts to quantify this influence of formaldehyde on soil solution pH. All presented data were corrected for these contaminations of ions and the pH influence.

2.3 Results

2.3.1 Sampling volume

The average sample volume of soil solution was 300 ± 100 μL for a distance of less than 1 mm from the root, 450 ± 150 μL between 1 and 5 mm from the root, 500 ± 150 μL between 6 and 10 mm from the root, and 600 ± 100 μL in the bulk soil. This decrease towards the roots reflected the influence of root water uptake on the water content of the soil.

Overall, sampling volume and concentrations of organic acid and inorganic anions did not reveal any significant relationship with Pearson r correlation coefficients lesser than -0.27. Figure 2.2a shows that nitrate concentrations were independent of the sampling volumes. On the other hand, we found higher concentrations of organic acids such as citrate at smaller sampling volumes (Figure 2.2b). The sampling volume was, however, also correlated with the distance to the nearest roots (Figure 2.2b), as is also shown above. The highest concentrations of citrate were found close to the root and these were also the samples with the smallest volume.
Figure 2.2: Relationship between sampling volume and concentration of nitrate (a) and citrate (b) in the rhizosphere of Lupinus albus at different distances from cluster roots.
2.3.2 Temporal variations of citrate in the rhizosphere of cluster roots

The release of substances from the roots to the rhizosphere refers to an exudation process. In the case of *Lupinus albus*, the exudation of organic acids will occur only during a limited period via the cluster roots because of their restricted life time.

Figure 2.3 shows the variation in citrate concentration of the rhizosphere solution of one cluster root over 11 days. For a period of 3 to 4 days, the citrate concentration in the region close to the cluster root quickly reached a maximum of sometimes up to 2 mM and then decreased again as rapidly as it rose.

During the exudation of citrate the pH of the rhizosphere soil solution dropped in average from 6.9 to 5.4 (Table 2.1). Figure 2.3 shows an example in which the pH around a single cluster root dropped from about 8 to 5.5 during exudation and stayed at the lower value for the rest of the sampling period.

![Figure 2.3: Temporal variation of citrate concentration and pH in the rhizosphere (<1 mm from cluster root) of Lupinus albus over 11 days. Result presented for one single cluster root.](image)
2.3.3 Comparison between rhizosphere and bulk soil solution

The composition of the soil solution samples differed significantly between the rhizosphere of *Lupinus albus* cluster roots and bulk soil (Table 2.1). The concentrations of citrate and malate were significantly higher near cluster roots than in the bulk soil. In contrast to the other organic acid anions, these two compounds were measured exclusively during the restricted time of exudation. The wide range of organic acid concentrations found in the vicinity of the cluster roots can be attributed to the exudation processes itself and the way we divided the sampling period into the three sub periods.

Table 2.1: Average concentration in μM (± standard deviation; range of concentrations for cluster roots during exudation) of low molecular weight organic acids, some inorganic anions and soil pH in bulk and rhizosphere soil solution of white lupin. For one parameter, different letters indicate significant differences between the values with Student t tests (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Rhizosphere</th>
<th>Cluster roots</th>
<th>Normal roots</th>
<th>Nodules</th>
<th>Bulk soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before exudation</td>
<td>During exudation</td>
<td>After exudation</td>
<td>n=8</td>
<td>n=6</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.2(0.9)a</td>
<td>394.6(3.8-2056.6)b</td>
<td>0.4(2.1)a</td>
<td>&lt;0.05</td>
<td>5.0(1.2)a</td>
</tr>
<tr>
<td>Oxalate</td>
<td>0.5(1.1)a</td>
<td>5.4(0.4-48.4)b</td>
<td>1.3(2.6)a</td>
<td>0.8(0.2)a</td>
<td>4.3(4.2)b</td>
</tr>
<tr>
<td>Malate</td>
<td>&lt;0.5</td>
<td>2.2(1.0-3.6)</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Acetate</td>
<td>3.2(0.4)a</td>
<td>24.5(2.1-369.3)c</td>
<td>17.0(24.6)c</td>
<td>11.1(2.2)b</td>
<td>52.2(28.6)d</td>
</tr>
<tr>
<td>Nitrate</td>
<td>32.3(45.2)b</td>
<td>41.9(69.4)b</td>
<td>21.0(56.5)a</td>
<td>27.4(21.0)b</td>
<td>14.5(19.4)a</td>
</tr>
<tr>
<td>Sulfate</td>
<td>5.2(5.2)a</td>
<td>5.2(20.8)a</td>
<td>11.5(30.2)a</td>
<td>32.3(35.4)b</td>
<td>11.5(4.2)a</td>
</tr>
<tr>
<td>Phosphate</td>
<td>57.9(64.2)b</td>
<td>58.9(78.9)b</td>
<td>29.5(49.5)a</td>
<td>68.4(14.7)c</td>
<td>56.8(8.4)b</td>
</tr>
<tr>
<td>pH</td>
<td>6.9(1.0)b</td>
<td>6.4(1.1)b</td>
<td>5.4(0.4)a</td>
<td>6.3(0.9)b</td>
<td>7.7(2.0)c</td>
</tr>
</tbody>
</table>

For the cluster roots, the sampling period was divided into the three sub periods “before”, “during” and “after” LMWOA exudation. “During” was defined as the period during which the concentration of citrate was increased 10-times above the background.

The class “during exudation” also includes those data from the beginning and the end of the exudation process with small concentrations. Considering that the maximal values during the exudative burst were generally high and could reach up to 2 mM, this has resulted in the very wide range of concentrations reported in Table 2.1.

Also, the concentrations of acetate and oxalate were significantly higher in the cluster root rhizosphere during the exudation. But low concentrations of these two acids were also found in almost all others samples. Acetate and oxalate were also higher near the nodules than in the
vicinity of normal roots and in the bulk soil. Although the number of samples was relatively small, it is very likely that the high acetate and oxalate concentrations near the nodules were related to the microbial activity in this symbiosis between plant and rhizobia.

Lower concentrations of nitrate and phosphate were found near the cluster roots following the exudation process compared to the bulk soil. No difference between bulk soil and cluster root rhizosphere was found for sulfate.

The rhizosphere pH of cluster root soil solution decreased following exudation and reached its lowest value measured in our experiment (Table 2.1).

Figure 2.4 shows the average spatial distribution of citrate around the 5 cluster roots sampled during our experiment. We observed a decrease in citrate concentrations with increasing distance from the cluster root.

![Figure 2.4: Average concentration of citrate at different distances from cluster roots. Average concentrations from 5 cluster roots during the exudation event are shown.](image)
2.4 Discussion and conclusion

Let us considering that a sampling volume of 300 and 500 \( \mu \text{L} \) corresponds to a sampled soil volume of about 600 and 1000 \( \mu \text{L} \), respectively, or a sampled soil cylinder around the suction cup with a diameter of about 1.2 or 1.6 cm, respectively. Then, the chemical composition of the samples represents about the average soil solution in this zone of influence. It thus consists of "true" rhizosphere soil solution in equilibrium with rhizosphere soil and roots but also soil solution that was transported from the bulk soil to the suction cup. If this soil compartment contains roots, these roots are "flushed" by the passing soil solution and exuded compounds are transported to the suction cup. The larger the sampling volume, the larger this influence becomes. Very small sample volumes on the other hand, allow for only a very restricted number of analyses and require very sensitive analytical methods, e.g., (Puschenreiter et al., 2005). High sampling rates as in our experiment are not only disadvantageous. First of all, the chosen water tension allowed for a good growth of lupin in the soil which was used in this study. Furthermore, sufficient sample volume for the various chemical analysis could be collected in a relatively short time period. Short sampling periods allow for high temporal resolution, e.g., to assess daily exudation cycles. Considering the different results from the relationship between sampling volume and concentration of nitrate and citrate in the rhizosphere of Lupinus albus at different distances from cluster roots we conclude that measured ion concentrations can effectively be related to root influence and that dilution effects can be neglected. However it is important to keep in mind that the sampling volume is also highly related to the soil conditions surrounding the sampling site. And in natural soil these conditions can be very heterogeneous and then have an influence on the differences observed between different sampling sites.

In this experiment, we observed, as it is well known from hydroponic studies, that citrate and malate are exuded by Lupinus albus (Dinkelaker et al., 1989; Gerke et al., 1994; Neumann et al., 2000). Moreover, the time course of the citrate exudation observed in our experiment is consistent with results from hydroponic cultures (Hagström et al., 2001; Skene, 2003; Watt and Evans, 1999). The rapid decrease of citrate following the exudative burst could be explained by consumption by microorganism, spatial diffusion in soil solution or sorption with soil matrix components (Jones, 1998; Jones et al., 2003).
Regarding those “non-exuded” organic acids (oxalate and acetate) found in higher concentration in the rhizosphere, we assume that an elevated microbial activity in the root zone has probably contributed to these results because microbes are responsible for the production of a wide range of organic acids especially in situations where nutrients may be limiting (Rozycki and Strzelczyk, 1986). As root exudation of organic acids has been found to play an important role in the mobilisation and uptake of nutrients (Jones, 1998; Marschner, 1995; Ryan et al., 2001) it was expected to observe lower concentration of nutrients mainly phosphate and nitrate in the rhizosphere of the cluster roots. There is ample evidence of water soluble P depletion occurring in the vicinity of the roots (Morel and Hinsinger, 1999). In addition, exchangeable phosphate liberated by exuded citrate is taken up at once. Furthermore nitrate is rapidly taken up in the rhizosphere due to the high demand for this essential macronutrient in plant nutrition (Jungk, 2002). As a difference, sulfate is only taken up at a low rate by plants (Marschner, 1995).

Release of organic acids as well as acidification of the rhizosphere have been found to affect the availability and uptake of mineral nutrient and this acidification can either be caused directly by the organic acids (Marschner, 1995) or by concomitant $\text{H}^+$-release (Jones, 1998).

This “rhizosphere effect” however is limited in the space. With the distance the root system influences will go rather in the opposite direction: organic acids concentration will decrease and inorganic anions concentration such as phosphate and nitrate will increase until it reaches the “bulk soil stage”. In our experiment, the decrease of component like citrate with distance from cluster root do not represent, however, the equilibrium soil solution composition in the respective distance from the roots, but about the average concentrations of citrate within the zone of influence of the suction cups discussed above. On the other hand, for the group of suction cups at a distance of 6 to 10 mm, for which the roots are just at the rim of the zone of influence, the citrate concentrations are still about a third of what is measured at a distance of $<1$ mm. This indicates some migration, either by diffusion or mass flow of exuded citrate away from the immediate vicinity of the rootlet apex despite consumption by microorganisms and adsorption to soil matrix components.

To conclude, we demonstrated that with our modified rhizobox micro suction cup system we were able to detect in-situ organic acids exuded from roots and to follow temporally an exudation process and its effect on soil solution chemistry. With this system we could also detect
differences between bulk soil, rhizosphere of cluster roots and rhizosphere of other roots (e.g., nodules). Moreover, to our best knowledge, this is the first time the exudation of organic acids by *Lupinus albus* has been shown *in-situ* in soil solution. Based on our results, the system is a good choice to study strong root exudation by single roots *in-situ*.

**Acknowledgements**

This work was supported by project C02.0084, State Secretariat for Research and Education, Switzerland, within COST Action 631 (Understanding and Modelling Plant-Soil Interactions In the Rhizosphere Environment) and the Fond Québécois de la Recherche sur la Nature et les Technologies. The rhizoboxes were designed and produced by Arthur Kölliker, Swiss Federal Institute for Forest, Snow, and Landscape Research (WSL).
2.5 References


Morel C and Hinsinger P 1999 Root-induced modifications of the exchange of phosphate ion between soil solution and soil solid phase. Plant Soil. 211, 103-110.


3 Spatial and temporal variation in organic acid anion exudation and nutrient anion uptake in the rhizosphere of *Lupinus albus* L.

Dessureault-Rompré J, Nowack B, Schulin R, Luster J

*Plant and Soil, in press.*

Abstract

We investigated *in-situ* the temporal patterns and spatial extent of organic acid anion exudation into the rhizosphere solution of *Lupinus albus*, and its relation with the nutrient anions phosphate, nitrate and sulfate by means of a rhizobox micro suction cup method under P sufficient conditions. We compared the soil solution in the rhizosphere of cluster roots with that in the vicinity of normal roots, nodules and bulk soil. Compared to the other rhizosphere and soil compartments, concentrations of organic acid anions were higher in the vicinity of cluster roots during the exudative burst (citrate, oxalate) and nodules (acetate, malate), while concentrations of inorganic nutrient anions were highest in the bulk soil. Both active cluster roots and nodules were most efficient in taking up nitrate and phosphate. The intensity of citrate exudation by cluster roots was highly variable. The overall temporal patterns during the lifetime of cluster roots were overlaid by a diurnal pattern, i.e. in most cases, the exudation burst consisted of one or more peaks occurring in the afternoon. Multiple exudation peaks occurred daily or were separated by one or two days. Although citrate concentrations decreased with distance from the cluster root apex, they were still significantly higher at a distance of 6 to 10 mm than in the bulk soil. Phosphate concentrations were extremely variable in the proximity of cluster roots. While our results indicate that under P sufficient conditions cluster roots take up phosphate during their entire life time, the influence of citrate exudation on phosphate mobilization from soil could not be assessed conclusively because of the complex interactions between P uptake, organic acid anion exudation and P mobilization. However, we observed indications of P mobilization concurrent with the highest measured citrate concentrations. In conclusion, this study provides semiquantitative *in-situ* data on the reactivity of different root segments of *Lupinus albus* L. in
terms of root exudation and nutrient uptake under nutrient sufficient conditions, in particular on the temporal variability during the lifetime of cluster roots.

3.1 Introduction

Several plant families have the ability to exude organic acid anions as a tolerance mechanism or as an adaptation to soils with low nutrient availability. *Lupinus albus*, a white-flowered Eurasian herb (Fabaceae-Leguminosae) that is widely cultivated for forage and also used for erosion control, is well known for its cluster roots that exude large amounts of citrate into the rhizosphere to cope with phosphate deficiency (Dinkelaker et al. 1995). Cluster roots are defined as a cluster of rootlets densely covered by root hairs that develop synchronously along a given length of a parent root (Dinkelaker et al. 1995; Marschner 1995; Skene et al. 1998). These specialized roots release citrate into the rhizosphere in a so-called exudative burst that is characterized by a sudden and marked release of high amounts of organic acid anions including citrate and malate as well as phosphatase and protons into the rhizosphere. This high exudative state lasts for 2-3 days before returning to a base level of exudation (senescence of the cluster root). This stage is followed by a decomposition of the cluster root after several weeks (Neumann and Martinoia 2002).

Citrate, one of the main compounds exuded by *Lupinus albus*, is a short-chain molecule with three carboxylic acid groups allowing the complexation of metal cations in solution and the displacement of anions from the soil matrix. This property explains the important role of organic acid anions in several soil processes such as mobilization and uptake of nutrients by plants and microorganisms or detoxification (e.g. Al tolerance) (Gerke et al. 2000; Hue et al. 1986; Jones 1998; Jones and Darrah 1994; Marschner 1995). The plant availability of P is limited to a large extent by the rate of the reaction that replenishes the pool of soluble P. The benefits of having organic acid anions in the rhizosphere are twofold: they compete with phosphate groups for binding sites in the soil, and they form stronger complexes with Al, Fe and Ca than phosphate (Ryan et al. 2001). Thus, they may help to release phosphate from inorganic phases by ligand exchange or ligand-enhanced dissolution (Johnson and Loeppert 2006).

The exudation of organic acid anions by *Lupinus albus* has been extensively investigated. Nevertheless, most of the studies have been performed using hydroponic systems (Neumann et al. 1999; Watt and Evans 1999b), by extraction of organic acid anions from rhizosphere soil
Chapter 3 Spatial and temporal variation in organic acid anion exudation

separated from the roots (Bayon et al. 2006; Dinkelaker et al. 1989; Gardner and Parbery 1982a; b; Gerke et al. 1994; Hagström et al. 2001; Li et al. 1997) or by comparing the composition of leachates from pot or soil columns experiments (Egle et al. 2003; Gardner et al. 1983a; Johnson et al. 1996; Shen et al. 2004; Shu et al. 2005).

However, roots in hydroponic systems can behave differently compared to natural conditions in soils (Marschner 1995) and the growth medium appeared to affect the cluster root formation (Peek et al. 2003; Shu et al. 2005; Watt and Evans 1999b). Furthermore, soil extracts do not allow temporal studies and leachates from pot and soil columns give information on a whole rhizosphere system without any precise spatial information. Thus there is a need to get a better understanding on the temporal variability of organic acid anion exudation by *Lupinus albus* cluster roots, and its spatial impact on nutrient availability in the cluster root rhizosphere solution as compared to the rhizosphere solution of other roots and bulk soil solutions.

The development of micro techniques for collection and analysis of soil solution has enabled micro-scale observation of soil solution chemistry (Göttlein et al. 1999; Göttlein et al. 1996; Vetterlein and Marschner 1993; Vetterlein et al. 1993). The use of micro suction cups in conjunction with rhizoboxes that allow to observe the development of root systems and sampling of the soil solution at defined distances from roots, has a large potential to study rhizosphere chemistry (Arocena et al. 2004; Dessureault-Rompré et al. 2006; Dieffenbach et al. 1997; Göttlein et al. 1999; Wang et al. 2004).

In our study we wanted to test the hypotheses (i) that different root segments of *Lupinus albus* differ in root exudation and their effect on soil solution composition, (ii) that exudation of organic acid anions by lupin cluster roots exhibits a diurnal variability, and (iii) during intensive exudation of organic acid anions by cluster roots phosphate is strongly mobilized. To this end, we investigated *in-situ* the temporal and spatial patterns of organic acid anions exuded by cluster roots of *Lupinus albus* and their relation to soil solution chemistry, in particular to nutrients such as phosphate, nitrate and sulfate by means of a rhizobox micro suction cup method (Dessureault-Rompré et al. 2006). In addition, we compared cluster root soil solution with soil solution composition in the vicinity of "normal" roots, nodules and in the bulk soil. In particular, no studies have been done so far according to our knowledge characterizing the soil solution composition surrounding nodules, which are of interest because of their symbiosis with rhizobia and their ability to fix atmospheric nitrogen.
3.2 Material and Methods

3.2.1 Rhizobox system

The rhizobox used in this study was described in detail by Dessureault-Rompré et al. (2006). It was adapted from the one introduced by Dieffenbach et al. (1997). We used a carbonate free soil (pH 6.4 (0.01 M CaCl₂), 15.1 g/kg Corg, 1.5 g/kg Ntot, 49 mg/kg Pavailable (Kuo 1996), 862 mg/kg Porg (Kuo, 1996), 36% sand, 49% silt, 15% clay). The soil was air dried, sieved (2 mm) and filled into the rhizoboxes at a bulk density of about 1.2 g/cm³. First, the rhizoboxes were rinsed with synthetic rainwater using the irrigation system described below operating at a positive head for 6 weeks (1 liter of leachate was collected from each rhizobox each week) in order to equilibrate the soil. Then the experimental conditions described below were established. Seeds of *Lupinus albus* ("Weissblühende Tellerlupine" cultivar, Ufa AG, Switzerland) were pre-treated with 10% hydrogen peroxide (Liang and Li 2003) and then germinated in black garden soil for one week. The plants were not inoculated with rhizobia but nodules were visible during the experimental period. Healthy plants were gently washed with deionised water to remove the organic black soil and transplanted into the rhizoboxes one week after establishing experimental conditions. Three rhizoboxes planted each with a single plant were used in this study.

The rhizobox experiment was conducted under controlled conditions in a climate chamber (light 16 h with an intensity at canopy height of 150 μm m⁻² s⁻¹, 80% humidity, temperature day/night: 20/16°C). The boxes were irrigated with synthetic rain water (ionic composition in μM: 70 NH₄, 70 NO₃, 3.2 PO₄, 17 Cl, 3.1 SO₄, 4.3 Na, 7.7 K, 5 Ca, 1.3 Mg, 0.15 Zn, pH =5.5) using wicks that were made from a polymer tube (Rhizon irrigators, Rhizosphere research products, Netherlands) and installed at 5, 30 and 55 cm soil depth. A hanging water column of 40 cm was maintained between each wick and a corresponding reservoir. With these settings the following water fluxes occurred within the rhizobox system. In plant free rhizoboxes, covered to minimize evaporation, the gravitational water flow from the upper two irrigation wicks to the lowest irrigation wick and the bottom of the rhizobox was measured to be approximately 15 ml day⁻¹. Assuming a porosity of 50%, this amounts to a downward flux of 2 cm day⁻¹. Evaporation from the soil surface, measured in uncovered rhizoboxes, was about 10 ml day⁻¹ or equal to an overall upward flux of 1.3 cm day⁻¹ (corresponding to 1.7 mm day⁻¹ of evaporation). In the planted rhizoboxes during the sampling periods, however, no outflow was observed. Total
irrigation volume was about 80 ml day\(^{-1}\) with each irrigation wick delivering roughly the same amount of around 25 ml day\(^{-1}\). This corresponded to about 20 ml of sampled solution plus an over-all evapotranspiration flux of about 60 ml day\(^{-1}\) or 10 mm day\(^{-1}\).

After the experiment, the shoots and roots of the plants were separated from each other. The root system was characterized in terms of parental root length as reference for the number of cluster roots. Then the plant material was dried at 65 °C and ground using a vibrating ball mill (Retsch MM 2000) equipped with an agate milling tool. The total chemical composition of shoots and roots were determined by ICP/OES analysis of acid micro wave digests.

### 3.2.2 Soil solution sampling

Samples were collected through the transparent front plate of the rhizoboxes as described by Dessureault-Rompré et al. (2006). The emergence of rootlets was the starting point for rhizosphere solution sampling around cluster roots. This happened between 4 and 7 weeks after sowing for the individual cluster roots, which were sampled for a period of 7 to 10 days, three times per day for 8 hours (6h-14h, 14h-22h, 22h-6h). The positioning of the micro suction cups allowed a sampling-free time at each position of 16 hours to reequilibrate the soil (Vetterlein and Jahn 2004). A total of nine micro suction cups were installed around a cluster root at three different distances to get spatial information (Figure 3.1). Each layer of micro suction cups included the 3 daily sampling periods. The three layers of micro suction cups (< 1 mm, 1-5 mm and 6-10 mm from the rootlet apex) were operated alternatively for 8 hours per day (6h-14h, 14h-22h, 22h-6h). The nine micro suction cups were installed in such a way that (i) the interaction between the individual cups was minimized, and (ii) their relative positions to the cluster roots were randomized. On 21 parental roots of about 60 cm length each, 11 cluster roots developed and were sampled (one cluster root per 115 cm parental root length; measured at the end of the experiment).
Figure 3.1: Distribution of micro suction cups around a cluster root. For each position the layer (L1, L2 or L3) and the beginning of the sampling period (06, 14, and 22 h) is indicated.

However, only the results from 7 cluster roots were included in the data analysis and interpretation. For the 4 remaining cluster roots, there were too many missing data due to insufficient material for analysis. During the 14-22h time periods, we also sampled the soil solution along 6 “normal” roots (NR), near 10 apices of “normal” roots (NRA), near 3 nodules (NOD) and at 15 bulk soil locations (BS; > 2 cm from the nearest root). For details on the sampling devices and sampling procedures we refer to Dessureault-Rompré et al. (2006).

3.2.3 Soil solution analysis

The volume was recorded for each soil solution sample. The samples were analyzed for low molecular weight organic acid anions (LMWOAA) (acetate, citrate, formate, lactate, malate, oxalate, propionate) and inorganic anions (sulfate, nitrate, and phosphate) using ion chromatography (Dionex autosampler system, AS 11 column, eluent: potassium hydroxide (1 to 60 mM), flow: 1.5 ml min⁻¹) with 200 μL insert glass vials to reduce the sample volume needed. Statistical differences for spatial and temporal data were tested using ANOVA. If p values indicated significant differences at a level of < 0.05, post hoc pairwise comparisons were carried out using Bonferroni/Dunn adjustment of probabilities. Analyses were carried out using SYSTAT 11.0.
3.2.4 Batch experiment to test phosphate mobilization from soil by citrate

Five grams of samples (oven-dried weight basis) of the same soil as used for the rhizobox experiment were shaken with 25 ml citrate solution (0, 500, 1000, 2500, 5000 μM) in a 50 ml polypropylene centrifuge tube for 24 hours at room temperature (~20°C). Formaldehyde solution was added at a concentration of 2 % to the citrate solution to prevent microbial degradation of the organic acid anions during the experiment. The soil suspension was centrifuged at 1060 g for 15 minutes and the supernatant filtered through Whatman Grade 602 h²/2. Phosphate and citrate in the extracts were analyzed using ion chromatography as describe above.

3.3 Results

3.3.1 Comparison between bulk and rhizosphere soil solutions

Table 3.1 shows the average concentrations of the measured anions in the soil solution of the different soil compartments sampled between 14 and 22h. Shown are the means and standard errors of the average values measured at the individual sampling positions. The average concentrations of citrate and oxalate in layer 1 of the cluster root rhizospheres were higher than in all other compartments. Citrate concentrations in the NRA and NOD rhizospheres were lower than in the CR rhizospheres but larger than in the NR compartment and in the bulk soil.

Malate concentrations were higher in all rhizosphere compartments compared to the bulk soil, but highest around nodules. Acetate showed a similar spatial distribution as malate. For propionate and lactate no significant differences were observed. However, there was a tendency for propionate to be higher near the nodules.

Phosphate and nitrate concentrations around cluster roots and nodules were lower than in all other compartments. When compared to the bulk soil, nitrate concentrations were also lower in the NRA and NR rhizospheres. Sulfate concentrations in the bulk soil were higher than in all rhizosphere compartments.
Table 3.1: Organic acid and inorganic anion concentrations (in µM) sampled at 14-22h in the rhizosphere solution of cluster roots (CR) in layer 1 (< 1 mm), apices of normal roots (NRA), normal roots (NR), and nodules (NOD) of Lupinus albus as well as in bulk soil solution (BS). Shown are the means and standard errors of the average concentrations at n individual sampling positions. In each row different letters indicate significant differences at p < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>CR (n=7)</th>
<th>NRA (n=10)</th>
<th>NR (n=6)</th>
<th>NOD (n=3)</th>
<th>BS (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>1231.3± 570.1 c</td>
<td>31.9± 12.4 b</td>
<td>10.9± 12.4 a</td>
<td>37.7±20.3 b</td>
<td>6.3±10.3 a</td>
</tr>
<tr>
<td>Oxalate</td>
<td>16.7± 12.0 b</td>
<td>4.2± 4.1 a</td>
<td>3.0± 6.4 a</td>
<td>5.4±3.9 a</td>
<td>3.2± 0.9 a</td>
</tr>
<tr>
<td>Malate</td>
<td>3.0± 0.4 b</td>
<td>1.7± 2.3 b</td>
<td>1.7± 1.5 b</td>
<td>7.1±2.8c</td>
<td>0.2±0.9 a</td>
</tr>
<tr>
<td>Acetate</td>
<td>54.0± 3.8 b</td>
<td>30.9±20.5 ab</td>
<td>19.4±10.0 a</td>
<td>153.2±63.2 c</td>
<td>25.3±19.4 a</td>
</tr>
<tr>
<td>Propionate</td>
<td>6.6±1.2 a</td>
<td>6.8±5.4 a</td>
<td>6.3±6.1 a</td>
<td>9.3±9.2 a</td>
<td>6.5±6.6 a</td>
</tr>
<tr>
<td>Lactate</td>
<td>29.1±1.9 a</td>
<td>35.2±12.1 a</td>
<td>33.4±9.2 a</td>
<td>27.5±12.3 a</td>
<td>33.6±11.6 a</td>
</tr>
<tr>
<td>Phosphate</td>
<td>70.9± 16.2 a</td>
<td>152.6± 8.3 b</td>
<td>163.2±15.1 b</td>
<td>109.4±25.3 a</td>
<td>184.2±73.3 b</td>
</tr>
<tr>
<td>Nitrate</td>
<td>33.1± 13.2 a</td>
<td>154.8± 19.9 b</td>
<td>101.6± 23.8 b</td>
<td>29.7±9.3 a</td>
<td>301.6±124.5 c</td>
</tr>
<tr>
<td>Sulfate</td>
<td>26.2± 4.6 a</td>
<td>26.0± 6.8 a</td>
<td>19.8± 5.4 a</td>
<td>15.7±7.5 a</td>
<td>45.8± 17.2 b</td>
</tr>
</tbody>
</table>

3.3.2 Temporal variability of organic acid anions in the cluster root rhizosphere

Figure 2 shows the rhizosphere concentrations of citrate during the lifetime of the seven cluster roots with a complete data set. The intensity and duration of the bursts of citrate differed considerably between individual cluster roots. Based on the data for layer 1, the behavior ranged from single exudation events (CR 2) to multiple exudation events separated by 1 (CR 1, 3, 4, 5), 2 (CR 7), or 3 days (CR 6). The concentration maxima occurred always in the time period from 14 to 22 h. The citrate concentrations in layer 3 were generally much smaller than in layer 1, but in most cases with an approximately similar temporal variation. The exception was CR 4, where during the second part of its life time high concentrations were observed in layer 3.

For most cluster roots, oxalate and malate concentrations in layer 1 varied irregularly at a low level (oxalate < 10 µmol L⁻¹; malate < 5µmol L⁻¹; data not shown). Only for the two cluster roots characterized by particularly high citrate exudation (CR 1 and 2) a more specific behavior could
be observed. Cluster root 2 was characterized by a pronounced maximum of malate of about 40 \( \mu \text{mol L}^{-1} \) during the same sampling period as the citrate maximum.

Oxalate, however, varied irregularly. For CR 1, the multiple citrate maxima were exactly mirrored by oxalate maxima of 50 to 300 \( \mu \text{mol L}^{-1} \), while only the second and fourth citrate maxima were coupled to maxima of malate of about 40 \( \mu \text{mol L}^{-1} \). Furthermore, both oxalate and malate exhibited additional maxima of similar intensity during the second part of the lifetime of CR 1 with no correspondence in citrate.

Figure 3.2: Temporal variability of citrate (upper part of panels) and phosphate (lower part of panels) concentrations in the layers 1 and 3 of all seven Lupinus albus cluster roots with a complete data set. For easier reference in the text, the cluster roots are numbered from CR1 to CR7.
3.3.3 Temporal variability of inorganic anions in the cluster root rhizosphere

Figure 3.2 also shows the rhizosphere concentrations of phosphate in layer 1 for all cluster roots with a complete data set. Generally, concentrations were lower than in the bulk soil solution (184 μM, dotted line). For most cluster roots (CR 2 to 7) phosphate exhibited a roughly similar behavior. Concentrations decreased somewhat or were low during the first part of the cluster root lifetime and increased slightly towards the end. The variability from sampling period to sampling period was small for CR 3, 5, and 7, while it was rather large for CR 2, 4, and 6. For CR 1, the very high citrate concentrations during the multiple exudation events were paralleled by elevated phosphate concentrations.

Figure 3.3 shows a plot of all citrate and phosphate data from layer 1 (<1 mm). No correlation between citrate and phosphate is visible. High P concentrations were observed both for low and high citrate concentrations and high citrate concentrations were often accompanied by low P concentrations. Moreover, two interesting observations can be made from the graph: concentrations of P higher than the bulk soil concentration are accompanied by intermediate citrate concentrations (10-1000 μM) and at higher citrate concentrations the tendency for low phosphate concentrations is greater.

Nitrate and sulfate concentrations in layer 1 varied irregularly without obvious temporal patterns (data not shown).
Figure 3.3: Relationship between citrate and phosphate concentrations in layer 1 (<1 mm) of cluster roots (open circles) and in the batch experiment (filled circles). The line shows the phosphate concentration in the bulk soil solution.

3.3.4 Spatial distribution of organic acid and inorganic anions in the rhizosphere of cluster roots

Figure 3.4 shows that, for all daily sampling periods, the average concentrations of citrate and oxalate in soil solution during the time period of maximum exudation decreased with increasing distance from the rootlets.
Figure 3.4: Spatial and diurnal variation of citrate (a), oxalate (b), nitrate (c) and phosphate (d) concentrations in the rhizosphere of cluster roots of Lupinus albus during the time of maximum activity beginning on the 6-14h sampling period before the first exudation peak and ending at the 22-6h sampling period after the last exudation peak. Shown are the means and standard errors of the average concentrations for all seven cluster roots with a complete data set stratified for sampling period and distance. Lower case lettering denote significant differences between different distances at the same sampling period, upper case lettering significant differences for different sampling periods at the same distance (n=7 for CR, N=15 for BS, p<0.05).

While nitrate and phosphate concentrations were significantly higher in the bulk soil than in the cluster root rhizosphere, there were no significant differences between the concentrations of these anions at different distances within the rhizosphere. The differences were statistically significant, however, only for citrate during the 14-22 h sampling period, when concentrations even at a distance of 6-10 mm from the rootlets were larger than in the bulk soil.
3.3.5 Mobilization of phosphate by citrate in batch experiments

The batch experiment showed that citrate was able to mobilize phosphate from the used soil. The concentration of mobilized phosphate was described by the following equation:

\[ P \,[\mu \text{mol L}^{-1}] = 0.114 \cdot \text{citrate (in equilibrium solution)} + 56.57 \,(R^2 = 0.992) \]

The data are also presented in Figure 3.3 (filled circles). Below 1.2 mM dissolved citrate there was little mobilization of phosphate but the phosphate concentration increased nearly 5 times at the highest input of citrate.

3.4 Discussion

3.4.1 Influence of sampling and system fluxes

Based on the observed water fluxes in the rhizoboxes one can conclude that during the sampling periods the major fluxes were the ones caused by transpiration and by sampling. Only at sampling positions in greater distance from roots, i.e. in the bulk soil or in layer 3 around cluster roots, some influence of gravitational flow can be expected. In the very vicinity of roots, it can be expected that, during the day, the matrix potential is smaller than applied at the irrigation wicks and gravitational flow is therefore smaller than measured in the plant free rhizobox. Together with the general considerations on the zone of influence for the sampling at a given position (Dessureault-Rompré et al. 2006), this has the following implications for the interpretation of the data: Measured concentrations should not be considered to quantitatively picture the situation at the place of sampling. They rather represent average conditions within a zone of influence that is given (i) by the sampling volume, (ii) by the content of water filled pores emptying within the range of matrix potentials applied at the suction cup during sampling, and (iii) by a directional preference governed by the transpirational and gravitational fluxes. Considering the dominant transpirational fluxes towards active root segments during the day, it can be expected that during this time all rhizosphere sampling positions should have a directional preference towards the bulk soil, i.e. the zone of influence is larger from the sampling position towards bulk soil than towards the root. As a consequence, the rhizosphere influence would
actually be underestimated, and the measured differences between bulk and rhizosphere soil and the extent of spatial gradients are actually larger than measured. At night, when transpirational fluxes were low, the directional preference was probably smaller. An influence of gravitational flow during this time – dilution of rhizosphere influence at positions above and enhancement at positions below cluster roots – would have been averaged out by the even distribution of spatial orientations of cluster roots.

With respect to the temporal resolution of the sampling around cluster roots, it has to be noted that an overlap of the zones of influence of positions sampled during different time periods could not be avoided completely because of the small dimensions of the cluster roots. Thus, sampling at a given position captured also water from a not sampled position within its zone of influence.

### 3.4.2 Root exudation by cluster roots

Our results show that the intensity of citrate exudation by cluster roots is highly variable, although some influence by the exact distance of layer 1 from the rootlets cannot be excluded. The overall temporal patterns during the lifetime of a cluster roots are overlaid by a diurnal pattern, i.e. in most cases, the exudation burst actually consists of one or more peaks occurring in the afternoon. Multiple exudation peaks can occur daily or be separated by one or two days.

The chance that hidden cluster roots are partly responsible for multiple peaks is small considering the following. (i) The size of the cluster roots was in the same range as the width of the rhizoboxes (1 cm) and the cluster roots formed dense rootlets with an average length of 5 mm. This spatial restriction reduced the possibilities of two cluster roots growing just one behind the other. (ii) The rhizosphere around cluster roots was sampled to a distance of up to 1 cm. The gradient of decreasing concentrations of citrate with increasing distance from the root indicated that only one single cluster root was sampled, because a second hidden cluster root would have to be exactly at the same position and orientation relative to the suction cups to cause such a spatial concentration pattern. An exception is CR 4. Here, the data from layer 3 suggest the appearance of a second cluster root nearby during the second half of its lifetime. (iii) The small spatial density of cluster roots, mentioned above, further reduced the chance of several cluster roots to be located very close to each other.
The mechanism by which exudation occurs is not well known (Watt and Evans 1999b), but it seems that carboxylate exudation may be sensitive to plant P status (Shane and Lambers 2005). Thus, if the exudation of citrate follows a diurnal pattern it should be in some way also linked to the P level of the plant. Major biochemical processes such as photosynthesis and respiration are activated by inorganic phosphate or its organic derivatives (Raghothama and Karthikeyan 2005). We can hypothesize that when the plant is highly physiologically active in the second period of the day, plant P level may decrease to a certain threshold thus stimulating a signal for the exudative burst. On the other hand, the high production and release of organic anions can be directly linked to the high photosynthetic activity during this period of the day.

It is generally assumed that concentrations of exuded organic acid anions in soils rapidly decrease with time and with increasing distance from the roots due to microbial consumption, adsorption to soil surfaces and diffusion in the soil solution (Jones 1998). On the other hand, Weisskopf et al. (2006) revealed several mechanisms by which Lupinus albus is able to reduce microbial activity during the most active period of a cluster root lifetime. The generally very strong diurnal variation during the exudation bursts suggests a rapid disappearance of the citrate exuded in the afternoon. The occurrence of significantly elevated citrate concentrations at a distance of 6-10 mm from the cluster roots at all daily sampling periods suggests, that the disappearance, apart from removal by sampling, microbial consumption, and adsorption to soil surfaces, can be attributed partly also to diffusive or convective transport away from the root.

Studies on spatial distribution of organic acid anions in soil are rare (Darrah 1991; Gardner and Boundy 1983; Gardner et al. 1983b; Hagström et al. 2001; Jones et al. 2003). Our results support the conclusions from early studies by Gardner and coworkers (1983, 1983a) on plants with cluster roots that there is a certain movement of the exuded organic acid anions in the soil. In an agar film experiment of Gardner et al (1983b) citrate released by proteoid roots was effective over considerable distances of at least 5 mm from the nearest root. Gardner and Boundy (1983) found that plants with cluster roots also enhance nutrient acquisition of other plants rooting in their vicinity. On the other hand, Jones et al. (2003) suspected that, in general, due to the very low diffusion coefficients of most organic acid anions in soil, the size of hot spots of organic acid anions released from the tip of a root hair, fungal hyphae or bacterial cell, may be only a few µm in diameter. The relatively large zone around lupin cluster roots influenced by
exudation may thus just be due to the high intensity of the exudative bursts and protective mechanisms inhibiting microbial consumption (Weisskopf et al. 2006)

Our results on organic acid anions other than citrate are not conclusive. To a large part this is probably due to the generally rather low concentrations and the respective analytical uncertainty. This is supported by the fact that more specific temporal patterns for oxalate and malate, however only partly mirroring citrate, were observed in cases where concentrations were rather high, i.e. for CR 1 and 2.

3.4.3 Root exudation and phosphate concentration in the rhizosphere soil solution of cluster roots

At the end of our experiment, the P level in the shoot of Lupinus albus was sufficient (500 \( \mu \)mol g\(^{-1}\) dry weight) as was the level of other macro and micro nutrients (Marschner 1995), which is in good agreement with the high concentrations of available soil P and the high P concentrations in the bulk soil solution. The observed temporal patterns of citrate exudation are valid for these conditions, but might be different under P stress conditions. From the literature it is known that the citrate exudation is inversely related to plant P status (Keerthisinghe et al. 1998). The variability of exudation between different cluster roots of one single lupin plant, however, has not received much attention so far. Nevertheless, it is somewhat surprising to observe concentrations of up to 13 mM of citrate in cluster root rhizospheres under P-sufficient soil conditions, in particular when considering the postulated dilution effect by sampling. On the other hand, it is known that the frequency and extent of P-deficiency can determine the spacing and length of rootlets along the proteoid root axis. As the severity of phosphorus stress increases, the proportion of the root system covered with proteoid rootlets increases (Watt and Evans 1999a). When P is absent from the growth medium of lupin, cluster roots may constitute more than 50% of the root dry mass compared to 5% when P is sufficient (Gilbert et al. 2000). The low density of 1 cluster root in 115 cm of parental roots in our rhizoboxes are in good agreement with the observations by Watt and Evans (1999a) and Gilbert et al. (2000) for P-sufficient conditions. Our results may thus suggest that the plant’s main adaptation to the P status of the soil is to adjust the spatial density of cluster roots rather than the amount of organic acid anions exuded from individual cluster roots. However, this needs further similar studies under different P conditions.
It is commonly believed that citrate is exuded to mobilize phosphate from the soil. The reaction of organic acid anions is expected to be highly dependent on soil type and thus mobilization of P is highly a soil specific reaction (Jones and Darrah 1994). Nonetheless, soil extraction experiments and theoretical calculations indicate that at least a millimolar carboxylate concentration in soil solution is required to achieve efficient P mobilization (Neumann and Martinoia 2002; Neumann and Römheld 2000; Penaloza et al. 2002). This is confirmed by the results of our batch experiment, which in turn indicates that our soil behaved like a "normal" soil regarding phosphate mobilization. This can explain why a correlation between exudation and P mobilization could only be observed for the extremely high citrate peaks of CR 1. Otherwise our data only suggest that under P sufficient conditions cluster roots take up phosphate during their whole lifetime. The irregular temporal variability of phosphate concentrations during the lifetime of the cluster roots may be explained (i) by the fact that they were governed mainly by two competing processes, root uptake and mobilization from the soil matrix by organic acid anions, (ii) by the influence of other root related processes such as the release of phosphatases and their impact on organic phosphate, and (iii) by spatial variability of mobilizable soil P.

### 3.4.4 Exudation and nutrient uptake by normal roots and nodules

The higher citrate concentrations in the NRA rhizosphere compared to the NR rhizosphere and the BS are in good agreement with the root apex being the most active part of a root. However, compared to the cluster roots the concentrations of organic acid anions attributed to root exudation by normal roots were quite small. A key difference between exudation by cluster and normal roots is related to the fact that cluster root exudation occurs at the same location whereas the main region of exudation by normal roots moves through the soil as the root elongates (Skene 2003). It is the combination of the cluster root capacity to release carboxylates in an exudative burst and their morphological structure that allows the build-up of high concentrations in the rhizosphere (Lambers and Colmer 2005).

In addition, our study provides some first in-situ characterization of the soil solution near root nodules. The presence of large amounts of acetate compared to other rhizosphere compartments is probably just indicative of the particularly high microbial activity in and around nodules. The elevated malate concentrations in the nodule rhizosphere may be related to the synthesis of malic
acid serving as intermediate compound in the production of C substrates for the nitrogenase in
the bacteroids (Marschner, 1995). Considering the function of nodules to supply the plant with
nitrogen by converting N₂ into ammonium, the intensive nitrate uptake by the nodules is probably
related rather to microbial consumption than to plant uptake.

3.5 Conclusions

From our in-situ soil solution study on citrate exudation by *Lupinus albus* cluster roots under P
sufficient conditions we conclude the following.

(i) The overall temporal patterns during the lifetime of cluster roots are overlaid by a diurnal
pattern, i.e. in most cases, the exudation burst actually consists of one or more peaks
occurring in the afternoon. Multiple exudation peaks can occur daily or be separated by
one or two days.

(ii) Our data are in favor of the notion that exuded citrate can diffuse in the soil solution to a
distance of more than 5 mm from the cluster root apices.

(iii) Mobilization of phosphate by citrate could not be assessed conclusively, probably because
of the complex interactions between P uptake, organic acid anion exudation and P
mobilization. On the other hand, our results indicate P mobilization concurrent with the
highest citrate concentrations encountered which supports earlier observations that only
very high citrate concentrations lead to a significant P mobilization.

Furthermore, our study demonstrated again the potential of the rhizobox micro suction cup
technique to study the influence of individual roots on soil solution chemistry. In particular, this
method seems to be well suited for a more detailed future study on rhizosphere effects around
root nodules.
Acknowledgements

This work was supported by project C02.0084, State Secretariat for Research and Education, Switzerland, within COST Action 631 (Understanding and Modelling Plant-Soil Interactions In the Rhizosphere Environment) and the Fond Québécois de la Recherche sur la Nature et les Technologies. The rhizoboxes were designed and produced by Arthur Kölliker, Swiss Federal Institute for Forest, Snow, and Landscape Research (WSL). Lena Püscher helped with the set-up of the rhizoboxes and the intensive sampling of soil solutions.
3.6 References


Gerke J, Römer W and Jungk A 1994 The excretion of citric and malic acid by proteoid roots of Lupinus albus L.; effects on soil solution concentrations of phosphate, iron, and


Watt M and Evans J R 1999a Linking development and determinacy with organic acid efflux from proteoid roots of white lupin grown with low phosphorus and ambient or elevated atmospheric CO₂ concentration. Plant Physiol. 120, 705-716.

4 Metal solubility and speciation in the rhizosphere of *Lupinus albus* L.

Submitted to *Environmental Science & Technology*

Abstract

The goal of this study was to investigate the influence of root exudation on nutrient and metal speciation in the rhizosphere of *Lupinus albus* using micro-suction cups. We found that large amounts of metals were mobilized in the rhizosphere of cluster roots during exudative bursts of citrate. DOC$_{uv}$ concentrations (dissolved organic carbon without organic acid anions) increased in parallel with organic acid anions (mainly citrate) concentrations. Speciation calculations revealed that Ca, Mn, Al and Zn were mainly complexed by citrate. The speciation of Cu and Pb was not affected by citrate and they were strongly complexed by DOC. Citrate complexed metals in the order Ca > Al > Fe > Mg > Mn. The investigations show that the effect of citrate exudation on metal solubility was twofold: On one hand metals like Zn, Fe and Al were directly mobilized and complexed by citrate and on the other hand citrate was mobilizing DOC$_{uv}$ which in turn then complexed and mobilized Cu and Pb.
4.1 Introduction

Root exudates have been implicated in several mechanisms for altering the solubility of ions and molecules in the rhizosphere in various ways (Cataldo et al. 1988). The amount and composition of root exudates is largely dependent in the nutritional status of the plant. Some species exude low-molecular-weight (LMW) organic acids in response to P and Fe deficiency or phytosiderophores in response to Fe and Zn deficiency (Haynes 1990; Jones and Darrah 1994). While some exudates can cause nutrients to be relatively more available for uptake by plants (Dakora and Phillips 2002), others allow plants to restrict uptake of toxic metals by the formation of non-toxic metal-ligand complexes in the rhizosphere. Such complexes may involve LMW organic acids, phosphate or high-molecular-weight polysaccharides exuded from roots (Ryan et al. 2001; Wenzel et al. 2001).

LMW organic acids, which occur as anions under a wide range of soil conditions, strongly bind metal ions in solution (Jones and Darrah 1994; Jones et al. 1996a). Many LMW organic acid anions (LMWOAA) are able to dissolve manganese and iron oxides or aluminum, calcium, and iron phosphates and liberate P for uptake by roots (Bolan et al. 1994; Dakora and Phillips 2002; Jones 1998; Jones and Darrah 1994; Marschner 1995). Furthermore, the LMWOAA can displace phosphate and sulphate from mineral surfaces by anion exchange (Bolan et al. 1994; Evans and Anderson 1990; Jones 1998; Jones and Darrah 1994). The reaction of LMWOAA with metals in soils however is dependent not only on the complexation ability of the LMWOAA but also on sorption/desorption reactions, and microbial degradation of the LMWOAA. Therefore, the degree to which the mobilization of metals by LMWOAA is achieved is highly dependent on the amount and type of LMWOAA released and on the physico-chemical and biological properties of the soil (Jones and Darrah 1994).

The impact of root exudates on metal concentrations in soil solution has been studied by means of soil extractions and adsorption experiments (Chen et al. 2003; Collins et al. 2003; Gao et al. 2003; Jones and Darrah 1994; Qin et al. 2004; Schwab et al. 2005) or pot experiments combined with soil extractions (Gerke et al. 1994). Most of these studies have looked at the effects of citrate on the desorption of metals from soil (Jones and Darrah 1994). Independent of the soil properties and concentration of LMWOAA used, they all observed an increased
desorption of these metals in the presence of LMWOAA. Moreover, changes in the rhizosphere chemistry during an exudation event are not only related to one specific LMWOAA but to the global exudation effect including microbial growth, as well as changes in pH and dissolved organic carbon (DOC) concentration.

The effect of root exudation on the DOC concentration in rhizosphere solution hasn’t received much attention so far. DOC plays an important role in the biogeochemistry of cationic nutrients and pollutants in soils because of chelation with polyvalent cations (Stevenson 1994). The exudation of organic compounds into the rhizosphere will increase the DOC concentration directly by the C-content of the exudates. Dessureault-Rompré et al. (2007) for example measured up to 9 mM citrate in the rhizosphere solution of *Lupinus albus*, which corresponds to 650 mg C L⁻¹. Also Zhao et al. (2007) measured in some samples in the rooted zone of willow an LMWOAA (malate and oxalate) contribution to DOC of 45-51%. LMWOAA can also increase DOC derived from soil organic matter (SOM), either indirectly via an increase of the microbial activity and the related SOM degradation or directly by anion exchange or complexation of Ca which is stabilizing SOM (Hauser et al. 2005; Yang et al. 2001).

The recent development of micro techniques for collection and analysis of soil solution has enabled the micro-scale observation of soil solution chemistry (Göttlein et al. 1999; Göttlein et al. 1996; Vetterlein and Marschner 1993; Vetterlein et al. 1993). The use of micro suction cups in conjunction with rhizoboxes that allow to observe the development of root systems and to sample soil solution at defined distances from roots has a large potential to study rhizosphere chemistry (Arocena et al. 2004; Dessureault-Rompré et al. 2006; Dieffenbach et al. 1997; Göttlein et al. 1999; Wang et al. 2004).

The objective of this study was to investigate the impact of root exudation of LMW OAA by *Lupinus albus* on the concentration and speciation of macro and micro elements in rhizosphere and bulk soil solution samples of unpolluted soil sampled by micro suction cups. *Lupinus albus* was chosen as a model plant to study such effects because it is well known to exude high amounts of citrate from specialized roots, the so-called cluster roots (Dinkelaker et al. 1995). Model calculations were used to assess the potential impact of the exudation on metal speciation in solution.
4.2 Materials and Methods

4.2.1 Rhizobox system

The rhizobox used in this study has been described in detail (Dessureault-Rompré et al. 2006). It is based on the design previously described. We used a carbonate free soil (pH 6.4 (0.01 M CaCl₂), 15.1 g/kg C<sub>org</sub>, 1.5 g/kg N<sub>tot</sub>, 49 mg/kg P<sub>available</sub> (0.5 M NaHCO₃, pH 8.5, Kuo, 1996) (Kuo 1996), 862 mg/kg P<sub>org</sub> (Kuo, 1996), 36% sand, 49% silt, 15% clay). The soil was air dried, sieved (2 mm) and filled into the rhizoboxes at a bulk density of about 1.2 g/cm³.

Seeds of Lupinus albus ("Weissblühende Tellerlupine" cultivar, Ufa AG, Switzerland) were pre-treated with 10 % hydrogen peroxide (Liang and Li 2003) and then germinated in black garden soil for one week. Healthy plants were gently washed with deionised water to remove the soil and then transplanted into the rhizoboxes. Three rhizoboxes planted each with a single plant were used in this study. Before the experiment the rhizoboxes were flushed with synthetic rain water for 6 weeks (1 liter of leachate was collected from each rhizobox each week) in order to equilibrate the soil.

The rhizobox experiment was conducted under controlled conditions in a climate chamber (light 16 h per day with an intensity at canopy height of 150 μm m⁻² s⁻¹, 80% humidity, temperature day/night: 20/16°C). The boxes were irrigated with synthetic rain water (ionic composition in μM: 70 NH₄, 70 NO₃, 3.2 PO₄, 17 Cl, 3.1 SO₄, 4.3 Na, 7.7 K, 5 Ca, 1.3 Mg, 0.15 Zn, pH =5.5) using wicks that were made from a polymer tube (Rhizon irrigators, Rhizosphere research products, Netherlands) and installed at 5, 30 and 55 cm soil depth. A hanging water column of 40 cm was maintained between each wick and a corresponding reservoir in order to establish an approximately constant matric potential of −40 hPa throughout the rhizobox. We refer to Dessureault-Rompré et al. (2007) for estimates of unsaturated hydraulic conductivity in this system and the possible small effects this may have on the interpretation of the results.

4.2.2 Soil solution sampling

Samples were collected through the transparent front plate of the rhizoboxes as described previously (Dessureault-Rompré et al. 2006). The emergence of rootlets was the starting point for rhizosphere solution sampling around cluster roots. This happened between 4 and 7 weeks after
sowing for the individual cluster roots, which were sampled for a period of 7 to 10 day, three times per day for 8 hours (6h-14h, 14h-22h, 22h-6h) to get information on different time periods. The positioning of the micro suction cups allowed a sampling-free time at each position of 16 hours to reequilibrate the soil (Vetterlein and Jahn 2004). For details on the sampling devices and sampling procedures we refer to Dessureault-Rompré et al. (2006). During the experiment, 11 cluster roots were sampled. This resulted in a total of 60 samples per diurnal sampling period. In addition we sampled soil solution in the bulk soil. During the 14-22h time periods, we also sampled the soil solution near 3 nodules (NOD) with 27 samples and at 15 bulk soil locations (BS) (> 2 cm from the nearest root) with a total of 100 samples. After the metal and LMWOAA analysis the remaining samples from cluster root rhizospheres were pooled to get samples with different citrate concentrations. The criterion for pooling the samples was to obtain different concentrations that represent the whole range of citrate concentration present in the rhizosphere solution. Five pools were made with citrate concentrations of 0.1, 569, 1147, 2378 and 9009 μM. These pools were used for voltammetric, UV and ICP-OES analysis. In addition, speciation calculations were performed for these pooled samples.

4.2.3 Soil solution analysis

Each soil solution sample was analyzed for LMWOAA (acetate, citrate, formate, lactate, malate, oxalate, propionate) and inorganic anions (nitrate, sulfate, and phosphate) using ion chromatography (Dionex autosampler system, AS 11 column, eluent generator: potassium hydroxide (1 to 60 mM), flow: 1.5 ml min⁻¹) with 200 μL insert glass vials to reduce the sample volume needed. UV absorbance was measured using a Varian Cary 50 spectrometer. DOC concentrations in the samples were estimated from the UV absorbance at 254 nm because the samples contained formaldehyde (CH₂O) added to prevent microbial degradation of the samples. Calibration of DOC vs. UV was performed using an extract of the same soil with synthetic rain water (composition see above) and determining the correlation between measured DOC (Total Organic Carbon Analyzer, Shimadzu autosampler, ASI-5000A) and UV absorption at 254 nm. This extract of soil used for the calibration contained negligible amounts of LMWOAA. We also considered the contribution of Fe-citrate complexes to the UV absorbance. We measured the UV absorbance of Fe-citrate solutions (from 0.2 to 1 mM Fe) and subtracted the contribution of
Fe-citrate assuming that all Fe in solution was complexed to citrate. Between 3 and 15% of total absorption could be attributed to Fe-citrate complexes. The reported DOC is considered exclusively soil derived, i.e. it does not include the contribution from citrate or other identified LMWOAAs, therefore this DOC is referred in the present chapter as DOC_{uv}. Total phenolics were analyzed in rhizosphere and batch experiment (section 3.2.4) samples with a colorimetric method according to Swain and Hillis (1959) using phenol as standard.

Total metal concentrations were analyzed in samples acidified at pH 2 using suprapur nitric acid (HNO₃) using ICP-MS (Na, Mg, Al, K, Ca, Mn, Fe, Ni, Cu, Zn, Cd, Pb) and ICP-OES (Fe and Ca if results from ICP-MS were above the measurement range). Square Wave Anodic Stripping Voltammetry (SWASV) using gel integrated microelectrode (GIME) arrays were also used for direct simultaneous measurements of Zn, Cd, Cu and Pb in raw samples. The GIME arrays consist of 100 interconnected Hg-plated Ir-based microdiscs, each of 5 μm diameter and a centre to centre spacing of 150 μm, covered by a 300 μm thick LGL Agarose gel (Tercier et al. 1995). Thanks to the characteristics of microelectrodes and of the gel membrane used, GIME-SWASV measurements in raw environmental samples at their natural pH allow sensitive (ppt level) and reliable detection of the dynamic fraction of Cu(II), Pb(II), Cd(II) and Zn(II), which is defined as the sum of the free metal ions and the small labile and mobile complexes with size of few nanometers (for detail, see Buffle and Tercier-Waeber 2000). Electrochemical measurements were performed by using a computer controlled Amel 433 potentiostat (Milan-Italy), coupled to a home-made preamplifier set at a value of 100, and a 5 ml Metrohm cell based on a three-electrode configuration, i.e.: a Metrohm Ag/AgCl/3 M KCl(sat) reference electrode integrated in a additional bridge of 0.1M NaNO₃ suprapur to avoid contamination of the test solution by metal present in the reference KCl(sat) electrolyte; a Metrohm platinum rod counter electrode; and the GIME working sensor described above. Hg deposition on the Ir-microdisks was performed, before each set of analysis, by applying a constant potential of -400 mV (vs Ag/AgCl/KCl sat./0.1M NaNO₃ reference electrode) in a deoxygenated solution of 5mM Hg(CH₃COO)₂ and 0.1 M HClO₄ (Belmont-Hébert et al. 1998) for 8 min. Reoxidation of mercury was carried out, at the end of each daily experiment, by scanning the potential linearly from -300 to +300 mV at 5mV/s in a deoxygenated 1M KSCN solution [b]. SWASV conditions used for trace metal measurements in soil samples and for calibration were as follows: cleaning time = 60 s; cleaning potential = -100 mV; deposition potential = -100 to 1200 mV; deposition
time = 0.5 to 3 min; frequency = 50 Hz; wave amplitude = 25 mV; step amplitude = 8 mV. Calibrations, by successive standard additions of the target metals, were performed in a 0.1 NaNO₃ suprapur solution.

pH was measured using an ion sensitive field effect transistor electrode (ISFET sensor, Sentron, The Netherlands).

### 4.2.4 Speciation calculations

The speciation of metals and LMWOAA in the rhizosphere and bulk soil solution was calculated using ECOSAT 4.8 (Keizer and VanRiemsdijk 2002) to determine the influence of LMWOAA and dissolved organic matter (DOM) on the speciation of metals. For the speciation calculations pH, the total concentrations of Mg, Al, Ca, Mn, Fe, Cu, Zn, Cd and Pb, and the concentrations of the anions Cl, NO₃, SO₄ and PO₄ were considered. Furthermore, the concentration of DOM was taken as twice the concentration of DOC and was subdivided into 40% humic acids, 40% fulvic acids and 20% unreactive DOC (Weng et al. 2002; Zhao et al. 2007). The complexation constants in the data base of the model were those given by (Lindsay 1979) and MinteqA2 (Allison et al. 1991).

### 4.3 Results

#### 4.3.1 Total dissolved metal concentrations during exudation events from cluster roots

The total concentrations of the analyzed metals increased sharply during the exudative burst of citrate and decreased again rapidly after citrate exudation ceased. Results are presented here for the exudation by one selected cluster root (Figure 4.1) for Al, Ca, Mn, Cu and Zn. For this exudation event the peak concentrations of the metals perfectly matched with the maximum concentration of citrate. It was also observed (data not shown) that the peak concentrations of metals were delayed of one sampling day after the peak exudation. The intensity of the exudative burst had a marked effect on the concentration of mobilized metals but the metal concentrations during the exudative burst were in all cases much higher than in the bulk soil. The pH in the
rhizosphere solution during the exudation events was in the neutral range and did not change importantly.

Figure 4.1: Citrate, pH and metal concentrations in rhizosphere solutions of cluster root of Lupinus albus during an exudation event. The dotted lines represent the bulk soil concentration. Asterisk (*) indicates concentrations below detection range (ICP-MS).

4.3.2 DOC\textsubscript{UV} mobilization in the rhizosphere solution

Figure 4.2 shows that the UV-absorption of the cluster root rhizosphere solutions increased with increasing citrate concentration. Citrate itself does not absorb significantly at 254 nm. Fe-citrate has a strong absorption at this wavelength but the contribution of Fe-citrate to the UV absorption at 254 nm was not more than 20%. The increase in UV-absorption must therefore be due to the direct or indirect mobilization of soil derived DOC\textsubscript{UV} by citrate. Total phenol concentration in the rhizosphere and in the soil extract from a batch experiment showed the same trend than the UV-absorption; it increased with citrate concentration.
Figure 4.2: Relationship between citrate concentration, total phenol concentration and UV absorption at 254 nm in pooled cluster root rhizosphere samples from Lupinus albus and in batch experiment (BE).

4.3.3 Metal speciation calculation and measurement in the cluster root rhizosphere solution

The results from the speciation calculations are in good agreement with the measured speciation. In particular, the concentration of the dynamic fraction of Cu(II), Pb(II), Cd(II) and Zn(II) measured by GIME-SWASV were found to be generally higher, within analytical errors, that the calculated free metal ion concentration (Figure 4.3).

The slopes $b$ of the relationship:

$$M_{\text{dynamic experimental}}[\%] = a + b \times M_{\text{free theoretic}}$$

are 2 for Cu ($R^2 = 0.89$), 1.5 for Pb ($R^2=0.91$), 1.3 for Cd ($R^2=0.95$), 1.2 for Zn ($R^2 = 0.87$) and, which show that the proportion of the labile and mobile metal complexes decrease in the
order Cu > Pb > Cd ≈ Zn. This is in good agreement with the general complexation properties of these trace metals.

Figures 4.4 shows, for pooled samples in bulk and rhizosphere solutions, the measured contributions of % dynamic (HM) and calculated free metal ions, metals complexed with LMWOAA and metals complexed with soil derived DOCuv. In the rhizosphere solution the free and % dynamic metals were in general lower than in the bulk soil (Figure 4.4). Because the total concentration of Cd in solution was often below the detection limit, the data basis is too small for any conclusive statement regarding this metal.
As shown in Figure 4.4 all metals except Cu, Pb and Cd were to some extent affected by the presence of LMWOAAs (mainly citrate) in the rhizosphere solution. For Mg in the rhizosphere, the contribution of citrate complexes was around 9% at this high citrate concentration. The citrate complexation of Al and Fe in the rhizosphere was over 80% and over 40%, respectively. In the rhizosphere solutions with this elevated citrate concentration, Ca was calculated to be complexed with citrate to about 60%, and Mn to about 80%. Zinc was a little more influenced by the presence of citrate and its complexation with citrate was around 40% in rhizosphere. For all metals, the degree of complexation with citrate was small in the bulk soil solution.
The presence of citrate also affected the complexation of metals by soil derived DOC_{UV} in the rhizosphere solution (figure 4.4). The lower complexation of Al, Mn, Fe, and Zn, by DOC_{UV} in the rhizosphere solution with elevated LMWOAA concentration compared to the bulk soil, occurred simultaneously with the increased complexation of those metals by LMWOAA. Only Mg complexation by DOC_{UV} generally was higher in the rhizosphere with high citrate concentration and as consequence, high DOC_{UV} concentration. Copper and Pb were complexed to around 90% by DOC_{UV}, independent of the concentration of LMWOAA or DOC_{UV} in the rhizosphere. Also Cd was mainly complexed with DOC_{UV} (80-90%). The complexation of Al and Fe with DOC_{UV} was much lower in the rhizosphere (<5%) and around 50% respectively compared to bulk soil Ca complexes with DOC_{UV} did not play an important role in neither case. Manganese was complexed with DOC_{UV} at around 10% in the rhizosphere compared to a complexation of around 40% in the bulk soil.

### 4.3.4 Citrate speciation

The calculated speciation of citrate in the cluster root rhizosphere is shown in Figure 4.5. The importance of metals for binding to citrate decreased in the following order: Ca > Al > Fe > Mg > Mn. There was also a large contribution of free citrate with 15 to 45% of total citrate.
4.3.5 Metal concentrations around nodules

Among the samples from the rhizosphere solution around nodules two distinct groups were identified: those with acetate concentrations only slightly elevated compared to the bulk soil (<50 μM) (representing 52% of the samples) and those with significantly elevated acetate concentrations (>50 μM) (representing 48% of the samples). The DOCUV concentration was about 25% higher in the solution with high acetate, but no statistics could be done due to the lack of replicates (Table 2). Magnesium, Al, Ca, Mn, Fe, and Zn were higher in the solution surrounding the nodule with high acetate than with low acetate (Table 4.2). This effect was statistically significant for Mn and Zn. The Cu concentration was significantly lower in the high acetate samples.

Table 4.1: Soil derived DOCUV, acetate and total metals in rhizosphere solution surrounding nodules, separated into samples with low (<50 μM) and high (>50 μM) concentrations of acetate.

<table>
<thead>
<tr>
<th></th>
<th>DOCUV*</th>
<th>Acetate</th>
<th>Al</th>
<th>Fe*</th>
<th>Ca*</th>
<th>Mg*</th>
<th>Mn</th>
<th>Zn</th>
<th>Cd</th>
<th>Cu</th>
<th>Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low acetate</td>
<td>1821</td>
<td>23.2a</td>
<td>4.76a</td>
<td>2.16</td>
<td>282.0</td>
<td>27.8</td>
<td>1.2a</td>
<td>0.21a</td>
<td>0.003a</td>
<td>0.7b</td>
<td>0.2a</td>
</tr>
<tr>
<td>(n=14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High acetate</td>
<td>2467</td>
<td>293.1b</td>
<td>11.99a</td>
<td>41.30</td>
<td>742.2</td>
<td>55.0</td>
<td>62.4b</td>
<td>1.47b</td>
<td>0.003a</td>
<td>0.4a</td>
<td>0.2a</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Statistical analysis was not possible for ICP-OES measurements of Fe, Ca and DOCUV in pooled samples

4.3.6 Calculated speciation of metals in the nodule rhizosphere solution

The speciation of metals in the rhizosphere solution of nodules differed clearly from the bulk soil solution and, in addition, depended on the acetate concentration. The main results are presented in Figure 4.6. For Zn and Cd the contributions of free metals were lower in the pooled rhizosphere solutions with high acetate concentration than in the one with low acetate concentration and in the bulk soil solution. The contributions of Zn and Cd complexed with DOCUV were higher in the rhizosphere solution with high acetate concentration than in the one with low acetate and in the bulk soil solution. Manganese was mainly bound by organic anions in the low acetate rhizosphere, while it occurred mainly as free Mn in the high acetate rhizosphere.
4.4 Discussion

4.4.1 Metal mobilization and complexation by LMWOAA exuded by Lupinus albus cluster roots

The exudation of LMWOAA by Lupinus albus cluster roots had a strong impact on the cation concentrations in rhizosphere solution. In average measured LMWOAA concentrations of 0.83 to 1.34 micromole (mostly citrate) were able to mobilize 1 μM of metals, predominantly Ca, Al and Fe. This is in good agreement with results presented by Jones and Darrah (1994) which indicated that citrate and malate are capable of mobilizing cations from alkaline soils but only a small amount is involved in micronutrient mobilization (Zn, Fe, Mn), while large amounts are involved in the mobilization of Ca and Mg (Jones and Darrah 1994). Although the complex formation constants of citrate, oxalate and malate with Al and Fe are much higher than with Ca (Ryan et al. 2001), the major part of citrate was complexed with Ca. This is due to the much better solubility of Ca compared to Fe and Al under the given soil conditions. Calcium concentrations were already high in the bulk soil and increased only to a relatively small extent (factor 2 to 3) as a consequence of citrate exudation, while the low Al and Fe concentrations in the bulk soil were increased by almost three orders of magnitude by the exudation of citrate. Manganese concentrations were increased by two orders of magnitude and Cu and Zn by a factor of about 5.
at maximum citrate exudation. The increased factors for Mn, Cu and Zn reflect the relative solubility of the metals at the given soil conditions. On the other hand, there was no effect of pH on the metal mobilization because it was stable in our experiment even during the highest citrate concentration. Therefore the increased concentration of metals observed in our experiment cannot be attributed to an acidification of the rhizosphere.

4.4.2 Relation between LMWOAA exudation by *Lupinus albus* cluster roots and DOC$_{UV}$ concentrations

The exudation of citrate caused a strong mobilization of soil organic matter resulting in high concentration of soil derived DOC$_{UV}$ in rhizosphere samples. The DOC$_{UV}$ could have been mobilized by different processes (Figure 4.7).

One of the important mechanisms by which citrate might release anions from soils is ligand exchange. As many authors confirm the capacity of LMWOAA like citrate to successfully compete with phosphate at the soil surface (Geelhoed et al. 1998; Violante et al. 1991) and also the role of phosphate in displacing DOM from sorption site (Beck et al. 1999; Kaiser and Zech 1997; Tipping 1981), citrate might increase the DOC$_{UV}$ concentration in solution directly by displacing surface-bound DOC$_{UV}$ from sorption sites. Indeed, the fact that we observed increased phenol concentration with high citrate concentration in the batch experiment may support this ligand exchange mechanisms.
Another mechanisms by which citrate might release anions such as DOC$_{UV}$ from soils is the ligand-enhanced dissolution of the oxide surface (Al, Fe, Ca oxides) (Johnson and Loeppert 2006). Although the dissolution of hydrous Fe oxides by OAA is well documented, its ecological meaning has received much less attention (Bertrand and Hinsinger 2000; Jones et al. 1996a). In soils and sediments clean oxides surfaces seldom exist and they are partly coated with organic matter (Gerin et al. 2003). Mikutta et al. (2006) observed that citrate can promote the partial dissolution of C-coated goethite by favoring the release of Fe(III)-organic complexes from the goethite surface. On one hand this mechanism may increase DOC$_{UV}$ and Fe in solution but on the other hand the mobilized organic compounds may complex other metal cations and thus the dissolution of Fe-oxides by citrate may induce the mobilization of both DOC$_{UV}$ and metals.

LMWOAA can also indirectly mobilize DOC$_{UV}$ from soils, presumably through complexation of Ca that is stabilizing the organic matter in the soil (dissolution of Ca-bridges) (Hauser et al.
According to our speciation calculations the free Ca concentration decreased from almost 100% at low citrate to about 40% at high citrate concentration. Although the total concentration of polyvalent metal cations was therefore higher during the exudation in the rhizosphere samples, the free concentration was lower and therefore SOM destabilization would have been possible through this mechanism.

However a counteracting mechanism may also reduce DOC\textsubscript{UV} solubility by aggregation with polyvalent cations. Increased concentrations of monovalent and polyvalent cations in solution might reduce DOC\textsubscript{UV} solubility. Surface complexes and ion bridges between negatively charged functional groups of DOC\textsubscript{UV} and cations in solution can reduce the surface charge density, alter the structural conformation of the DOC\textsubscript{UV} and consequently reduce solubility caused by coagulation (cation bridging) and flocculation (Kalbitz et al. 2000). This could in fact explain the sudden disappearance of DOC\textsubscript{UV} after the citrate exudation ceased.

Finally it is also possible that the increase of soil derived DOC\textsubscript{UV} in the rhizosphere may be an indirect effect through an increase of the microbial biomass and hence the microbial activity. Higher enzymatic activity caused by the increased microbial activity can efficiently attack organic matter and release DOC\textsubscript{UV} into solution (Wengel et al. 2006). Bacteria and fungi readily take up and metabolize organic acids (Jones et al. 1996b; Strom et al. 2001; vanHees et al. 2005). However it has been shown by Weisskopf et al. (2006) that white lupin has developed a complex strategy to reduce microbial degradation of its root exudates by a decrease in pH and the release of antifungal compounds (isoflavonoids and wall-degrading enzymes). In our experiment no pH decrease was observed.

### 4.4.3 Combined effect of LMWOAA exudation and DOC\textsubscript{UV} mobilization on metal mobilization in the *Lupinus albus* cluster root rhizosphere

The exudation of citrate resulted in a complex network of reactions that led to the mobilization of DOC\textsubscript{UV}, major cations (e.g. Ca) and metals (e.g. Fe, Al, Zn, Cu). Although the metals Cu, Zn, Cd and Pb only make up a very small fraction of the citrate complexes, these metals are strongly influenced by exudation. Again various mechanisms can cause such mobilization. Metals like Zn, Fe and Al are directly mobilized and complexed by citrate. According to the speciation calculations a significant fraction of these metals was bound to citrate during the exudation event.
On the other hand there is also an indirect effect of citrate through mobilization of DOC$_{UV}$ which in turn is then complexing and mobilizing metals such as Cu and Pb.

A possible reason for the disappearance of the metals in solution at the same time as citrate exudation ceases exist in the counteracting mechanisms discussed above. Because of the high concentration of cations in solution, chemical reactions between anionic functional groups of organic molecules and cations might lead to the reduction of surface charges, coagulation and flocculation of DOC$_{UV}$ together with cations (Kalbitz et al. 2000), thus decreasing both cations and DOC$_{UV}$ concentrations in solution.

### 4.4.4 Nodules

To our knowledge no previous studies have been published characterizing the soil solution composition surrounding nodules and especially the influence of these microstructures on metal mobilization. Root nodules are formed on the roots of plants that associate with symbiotic N-fixing bacteria. The fixation of atmospheric nitrogen and its reduction to NH$_3$ is highly endergonic and cause the environment surrounding the nodule to be a highly reductive one. The energy consume in the nodule derives from sucroses directly from the phloem of the plant host. In legume species such as lupin the amino acid asparagine is the main compound exported from the nodules into the xylem and the shoot (Marschner 1995). Asparagine enters the citric acid cycle where it is metabolized into oxaloacetate. The reason that we have measured high acetate concentrations in the rhizosphere solution of the nodule remains unclear. The presence of large amounts of acetate compared to other rhizosphere compartments may also be just an indicator of the particularly high microbial activity in and around nodules. Whatever is the reason for the difference in acetate, the rhizosphere samples with low and high acetate concentration showed clear differences in total metal solubility and metal speciation. These results warrant a further systematic investigation of this very active rhizosphere compartment.
Acknowledgements

This work was supported by project C02.0084, State Secretariat for Research and Education, Switzerland, within COST Action 631 (Understanding and Modelling Plant-Soil Interactions In the Rhizosphere Environment) and the Fond Québécois de la Recherche sur la Nature et les Technologies. The rhizoboxes were designed and produced by Arthur Kölliker, Swiss Federal Institute for Forest, Snow, and Landscape Research (WSL). Lena Püschel helped with the set-up of the rhizoboxes and the intensive sampling of soil solutions.
Chapter 4 Metal solubility and speciation in the rhizosphere

References


Chapter 4 Metal solubility and speciation in the rhizosphere


Keizer, M.G., VanRiemsdijk, W.H. 2002 A computer program for the equilibrium calculation of speciation and transport in soil-water systems (ECOSAT 4.7), Department of soil quality, Wageningen University.


Lindsay, W.L. 1979 Chemical equilibria in soils, Wiley Interscience, New York.


Swain, T and Hillis WE. 1959. The phenolic constituents of Prunus domestica. 1 The quantitative analysis of phenolic constituents. J. Sci. Food. Agric. 10, 63-68.


5 Mobilization and complexation of Zn and Cd in the rhizosphere of *Thlaspi caerulescens*

Dessureault-Rompré J, Nowack B, Schulin R, Marie-Louise Tercier-Waeber, Luster J
Submitted to *Environmental Pollution*

Abstract

The goal of this study was to investigate the mobilization and complexation of Zn and Cd in the rhizosphere of different *Thlaspi* species and ecotype, and their change over time. Our hypotheses were that *Thlaspi caerulescens* can release root exudates in its rhizosphere to enhance the mobilization and complexation of Zn and Cd and that there is difference between different ecotype and species. Using a modified rhizobox system, we found that the total Zn concentration in solution slightly increased with time in the rhizosphere of the ecotypes *Thlaspi caerulescens* Prayon and Gange. These results agree with the possibility of a mobilization of Zn and Cd in the rhizosphere solution of *Thlaspi caerulescens*. The dynamic fraction of Zn and Cd decreased considerably over time in the rhizosphere solution of Gange and Prayon (only Zn), and were at the end of the experiment about 30 to 60% less compared to *Thlaspi perfoliatum* and the plant-free soil. These results indicate that it is not primarily mobilization that is involved in the hyperaccumulation mechanism of Zn and Cd by *Thlaspi caerulescens* but that the complexation of those metals is influenced by the plants. The potential ligands in the rhizosphere of Ganges and Prayon are selective for Cd and Zn and do not change Cu and Pb speciation. UV absorbance spectroscopy results revealed that the rhizosphere solutions of *Thlaspi caerulescens* Gange and Prayon were characterized by higher specific absorbance compared to *Thlaspi perfoliatum* and the plant-free soil.
5.1 Introduction

Hyperaccumulators are plants exhibiting an extraordinary capacity to accumulate heavy metals in their shoots (Baker and Brooks 1989). In recent years they have been extensively studied because of their potential use in phytoextraction of metals from polluted soils. *Thlaspi caerulescens* J. and C. Presl (Brassicaceae) is one of the best known hyperaccumulator plants (Lombi et al. 2000; Robinson et al. 1998; Schwartz et al. 2003; Zhao et al. 2003). This species has been shown to hyper accumulate Cd up to 2000 mg kg\(^{-1}\) and Zn up to 40000 mg kg\(^{-1}\) in shoot dry matter without showing any toxicity symptoms (Reeves et al. 2001). However while *Thlaspi caerulescens* has an extraordinary capacity to accumulate Zn and Cd in the shoots, the efficiency in removing these metals from the soil is limited due to the low biomass of the plants. Model calculations and field experiment suggested that phytoremediation using *T. caerulescens* may be feasible when the soil is only moderately contaminated with Cd and Zn (McGrath et al. 2006; Zhao et al. 2003). Field experiments have shown that the remediation of Cd contaminated soil by *Thlaspi caerulescens* would take between 9 and 49 years in a moderately contaminated soil (Hammer and Keller 2003) depending on soil properties. Because bioavailability of metals depends to a great extent on the speciation in the solid phase and the solution (Plette et al. 1999) soil properties such as pH, redox equilibria, organic matter and clay content may influence greatly the uptake of metal by plants. Metals can exist in different forms in soils ranging from highly labile species which are readily bioavailable to increasingly non-labile fixed forms. Under certain conditions such as change in the rhizosphere chemistry (pH, redox potential, presence of complexing agent...) the fixed metals can be released into the labile pool.

Numerous attempts have been made to identify the mechanism of hyperaccumulation by *Thlaspi caerulescens* and its impact on metal availability in the rhizosphere. To date no experimental evidence was found to support a mobilizing mechanism (Whiting et al. 2001a) and isotopic dilution studies showed that hyperaccumulator plants access the same pool of metals than other plants (Ayoub et al. 2003; Gérard et al. 2000; Hammer et al. 2006; Hutchinson et al. 2000; Schwartz et al. 2003). However, because of a large Zn and Cd uptake, the kinetics of replenishment of the depleted bioavailable pool may lead to depletion of pools thought to be otherwise poorly available to plants (Hammer and Keller 2002; Keller et al. 2001).
Several studies have compared hyperaccumulators and non-accumulators to assess changes in rhizosphere chemistry parameters that could result in solubilisation of non-labile forms of metals, but failed to find a correlation between metal uptake and root-induced changes in the rhizosphere such as acidification and release of complexing agent (Knight et al. 1997; McGrath et al. 1997; Zhao et al. 2001). Only two mechanisms are found to distinguish Zn and Cd uptake by *Thlaspi caerulescens*: the higher Zn and Cd transporters density in root cells (Lombi et al. 2001; Pence et al. 2000) and an active proliferation of root branches towards Zn/Cd-rich patches (Haines 2002; Schwartz et al. 1999; Whiting et al. 2000).

On the other hand, the high root density of *T. caerulescens* may enhance the highly efficient uptake into the roots coupled with replenishment of the Zn taken up through soil buffering capacity (Whiting et al. 2001b). Keller et al. (2003) showed in a field experiment that a large cumulative root density/above ground biomass ratio together with a larger proportion of fine roots compared to other plants, seemed to be additional favourable characteristics to increase metal uptake by *Thlaspi caerulescens*. The specific development of the root system towards patches with high metal concentrations is probably a primary factor which controls the efficient removal of excess metals, this proliferation is particularly advantageous because of heterogeneity of the distribution of metal in polluted soils (Schwartz et al. 1999). Ingwersen et al (2006) concluded from a micro-lysimeter and modelling experiment that *T. caerulescens* accelerates the resupply of Cd from soil, pointing to an important role of kinetic desorption in the hyperaccumulation by this plant.

Many different approaches have been used to study the mechanisms of metals hyperaccumulation by *Thlaspi caerulescens*. Most of the studies have used pot experiment with plant and soil analysis. Few have used soil solution analysis (Ingwersen et al. 2006; Keller and Hammer 2004; Knight et al. 1997; Luo et al. 2000; Yanai et al. 2006) and most of the studies analysed total metal concentrations in soil or soil solution. Only a few estimated metal bioavailability using technique such as isotopic dilution or selected extraction. Few attempted to compare labile or free metal fractions with total concentration (Knight et al. 1997) or to distinguish experimentally between rhizosphere and bulk solution. The development and use of precise analytical tool such as square wave anodic stripping voltammetry using microelectrode arrays (Buffle and Tercier-Waeber 2000) has provided tools to analyse labile metal fractions. In combination with the development of micro sampling techniques this opened new ways to study
soil solution chemistry (Göttlein et al. 1999; Göttlein et al. 1996; Vetterlein and Marschner 1993; Vetterlein et al. 1993). In conjunction with rhizoboxes that allow to observe the development of roots micro suction cups can be used to sample soil solution at defined distances from roots and thus to analyze the dynamics of chemical gradients between bulk and rhizosphere solutions (Arocena et al. 2004; Dessureault-Rompré et al. 2006; Dieffenbach et al. 1997; Göttlein et al. 1999; Wang et al. 2004).

Our objective was to investigate the heavy metal solubility and complexation in the rhizosphere solution of *Thlaspi caerulescens*. Our hypotheses were that *Thlaspi caerulescens* can release root exudates in its rhizosphere to enhance the mobilization and complexation of Zn and Cd and that there is difference between different ecotype and species. We used two well studied ecotypes of *Thlaspi caerulescens* growing on metalliferous soils: Ganges hyperaccumulating Zn and Cd and Prayon hyperaccumulating only Zn. *Thlaspi perfoliatum* was also used as a control plant that does not show hyperaccumulating traits (Reeves et al. 2001). The study was conducted by means of an *in situ* rhizobox micro suction cup method (Dessureault-Rompré et al. 2006).

### 5.2 Material and Methods

#### 5.2.1 Rhizobox system

We used an agricultural topsoil (Caslano, Switzerland) (FAO: Fluvisol) onto which wastes from septic tanks had been applied regularly between 1960 and 1980. The soil was a weakly sandy loam acid (pH$_{H_2O}$ 5.15). The organic carbon content was rather high (66 g/kg C$_{org}$), due to the waste application. The total N content and the CEC were in the normal range for a Swiss agricultural topsoil (2 g/kg N$_{tot}$, CEC 163 mmol kg$^{-1}$, 68% sand, 20% silt, 12% clay). The total concentrations of Cu, Zn, and Cd in the soil were 227, 666 and 2.49 mg/kg, respectively. The soil was air dried, sieved (2 mm) and filled into the rhizoboxes at a bulk density of about 1.2 g/cm$^3$.

Different *Thlaspi* species were compared in this study: two ecotypes of *Thlaspi caerulescens* J. & C. Presl. (Brassicaceae) i.e. the Cd and Zn hyperaccumulator “Gange” (les Avinières, St-Laurent-le-Minier, France) (Robinson et al. 1998) and the Zn hyperaccumulator “Prayon” (Prayon, Belgium). For control, the non-hyperaccumulator *Thlaspi perfoliatum* (L.) F.K. Meyer (Jura, Bern Canton) was used. All plants were germinated and grown in commercial garden soil for 52
days. Healthy plants were gently washed with deionised water to remove the commercial soil and then transplanted into rhizoboxes.

The rhizobox used in this study has been described in detail by Dessureault-Rompré et al. (2006). It was adapted from the one introduced by (Dieffenbach et al. 1997). Nine rhizoboxes (3 replicates for each Thlaspi plant) were planted each with a single plant. In addition we used three rhizoboxes with soil only as plant-free control (PFS). Before the experiment started the rhizoboxes were flushed with synthetic rain water for 6 weeks (1 liter of leachate was collected from each rhizobox each week) in order to equilibrate the soil. We define the treatment effect in our experiment as the difference between Thlaspi caerulescens Gange ecotype, Thlaspi caerulescens Prayon ecotype, Thlaspi perfoliatum and the PFS.

The rhizobox experiment was conducted under controlled conditions in a climate chamber (light 16 h with an intensity at canopy height of 150 μm m⁻² s⁻¹, 80% humidity, temperature day/night: 20/16°C). The boxes were irrigated with synthetic rain water (ionic composition in μM: 70 NH₄, 70 NO₃, 3.2 PO₄, 17 Cl, 3.1 SO₄, 4.3 Na, 7.7 K, 5 Ca, 1.3 Mg, 0.15 Zn, pH =5.5) using wicks that were made from a polymer tube (Rhizon irrigators, Rhizosphere research products, Netherlands) and installed at 5, 30 and 55 cm soil depth. A water potential of −40 hPa was applied by means of a hanging water column between the wicks and the reservoir.

5.2.2 Soil solution sampling

Samples were collected through the transparent front plate of the rhizoboxes using micro suction cups as described by Dessureault-Rompré et al. (2006). Rhizosphere solution sampling started in week 10 after sowing (WAS) once a week for 6 hours during the day light period and was performed until week 22 (WAS 22). Each week micro suction cups were positioned in the rhizoboxes in a way to follow the growing roots and at a distance < 1mm of the root. Each week some micro suction cups were removed from the rhizoboxes while new ones were added close to the most active roots (root apices and bright white roots). The number of micro suction cup positioned increased with root density from around 5 micro suction cups per rhizobox at the beginning of the experiment to around 10-20 from WAS 16 depending of the plant. All samples collected from the same rhizobox were pooled in one or two samples. Thus we obtained a minimum of 3 replicates per sampling week and per plant species and ecotypes. The same
procedure was carried out once a week (there was two sampling per week) with addition of formaldehyde to each sampling syringe in order to inhibit microbial degradation of low molecular weight organic acid anions (LMWOAA) in the samples. Each week the root development in each rhizoboxes was recorded visually and by photographs (length of the roots, root density comparison between the rhizoboxes etc.)

5.2.3 Soil solution analysis

All samples were analyzed for the low molecular weight organic acid anions (LMWOAA) acetate, citrate, formate, lactate, malate, oxalate, propionate and inorganic anions sulfate, nitrate, and phosphate using ion chromatography ( Dionex autosampler system, AS 11 column, eluent generator: potassium hydroxide (1 to 60 mM), flow: 1.5 ml min⁻¹). Insert glass of 200 µL were used to reduce the required sample volume. UV absorbance was measured between 200 and 700 nm using Varian Cary 50 spectrometer. Dissolved organic carbon (DOC) was determined using a Total Organic Carbon Analyzer (Shimadzu autosampler, ASI-5000A). Total metal concentrations were analyzed in samples acidified at pH 2 using suprapur nitric acid (HNO3) using ICP-MS (Na, Mg, Al, K, Ca, Mn, Fe, Ni, Cu, Zn, Cd, Pb) and ICP-OES (Fe and Ca if results from ICP-MS were above the measurement range). Square Wave Anodic Stripping Voltammetry (SWASV) using gel integrated microelectrode (GIME) arrays were also used for direct simultaneous measurements of Zn, Cd, Cu and Pb in raw samples. The GIME arrays consist on 100 interconnected Hg-plated Ir-based microdiscs, each of 5 µm diameter and a centre to centre spacing of 150 µm, covered by a 300 µm thick LGL Agarose gel (Tercier et al. 1995). Thanks to the characteristics of microelectrodes and of the gel membrane used, GIME-SWASV measurements in raw environmental samples at their natural pH allow sensitive (ppt level) and reliable detection of the dynamic fraction of Cu(II), Pb(II), Cd(II) and Zn(II), which is defined as the sum of the free metal ions and the small labile and mobile complexes with size of few nanometers (for detail, see Buffle and Tercier-Waeber 2000). Electrochemical measurements were performed by using a computer controlled Amel 433 potentiostat (Milan-Italy), coupled to a home-made preamplifier set at a value of 100, and a 5 ml Metrohm cell based on a three-electrode configuration, i.e.: a Metrohm Ag/AgCl/3 M KCl(sat) reference electrode integrated in a additional bridge of 0.1M NaNＯ₃ suprapur to avoid contamination of the test solution by metal
present in the reference KCl(sat) electrolyte; a Metrohm platinum rod counter electrode; and the GIME working sensor described above. Hg deposition on the Ir-microdisks was performed, before each set of analysis, by applying a constant potential of $-400 \text{ mV (vs Ag/AgCl/KCl sat/0.1M NaNO}_3\text{ reference electrode)}$ in a deoxygenated solution of 5mM Hg(CH\text{3COO})_2 and 0.1 M HClO\text{4 (Belmont-Hébert et al. 1998) for 8 min. Reoxidation of mercury was carried out, at the end of each daily experiment, by scanning the potential linearly from -300 to +300 mV at 5mV/s in a deoxygenated 1M KSCN solution \text{[b]. SWASV conditions used for trace metal measurements in soil samples and for calibration were as follows: cleaning time = 60 s; cleaning potential = -100 mV; deposition potential = -100 to 1200 mV; deposition time = 0.5 to 3 min; frequency = 50 Hz; wave amplitude = 25 mV; step amplitude = 8 mV. Calibrations, by successive standard additions of the target metals, were performed in a 0.1 NaNO\text{3 suprapur solution.}}$

The pH was measured using an ion sensitive field effect transistor electrode (ISFET sensor, Sentron, The Netherlands).

Statistical differences between treatments and the time effects were tested by means of a 1-way ANOVA with significant differences at a $p$ value of $<0.05$. Analyses were carried out using SYSTAT 11.0.

5.2.4 Complexation studies based on the dissociation of metal complexes

Residual fractions of the solution samples from the experiment were pooled by treatments in order to obtain a large and representative sample for each treatment. Each of them was subdivided into subsamples and the pHs was varied from 7.0 to 2.0 to obtain information on the complexation strength for Cu, Zn, Cd, and Pb. The pH values were fixed in the following way:

In the pH range from 5.5 to 7.0 the pH values were fixed by means of $10^{-2}$ M analytical grade MES ((2-(N-morpholino)-ethane sulfonic acid)) buffer solutions, with pH adjusted by suprapur nitric acid and sodium hydroxide. In the pH range 4-5, the pH was fixed by means of $2\times10^{-2}$ M suprapur acetate buffer solution, with pH adjusted by suprapur nitric acid and sodium hydroxide. In the pH range 2-3.5, the pH was adjusted by adding HNO\text{3. The background concentrations of Cu(II), Zn(II), Cd(II) and Pb(II) in the MES and acetate buffer were checked by blank measurements and no corrections was done because the concentrations were negligible compared to the soil solution concentrations. Each solution was equilibrated for at least 3 days and the pH
was checked regularly. For each pH dynamic Cu, Zn, Cd, and Pb concentrations in solution were
analyzed by square wave anodic stripping voltammetry using microelectrode arrays as describe
before (Buffle and Tercier-Waeber 2000).

5.2.5 Speciation calculations

The speciation of metals and OAA in the rhizosphere and bulk soil solution was calculated
using ECOSAT 4.8 (Keizer and VanRiemsdijk 2002) to determine the influence of OAA and
dissolved organic matter (DOM) on the speciation of metals. For the speciation calculations pH,
the total concentrations of Mg, Al, Ca, Mn, Fe, Cu, Zn, Cd and Pb, and the concentrations of the
anions Cl, NO₃, SO₄ and PO₄ were considered. Furthermore, the concentration of DOM was
taken as twice the concentration of DOC and was subdivided into 40% humic acids, 40% fulvic
acids and 20% unreactive DOC (Weng et al. 2002; Zhao et al. 2007). The complexation constants
in the data base of the model were those given by (Lindsay 1979) and MinteqA2 (Allison et al.

5.2.6 Plant metal concentrations

After the experiment, the shoots and roots of the plants were separated from each other. Then
the plant material was dried at 65 °C and ground using a vibrating ball mill (Retsch MM 2000)
equipped with an agate milling tool. The total chemical composition of shoots and roots were
determined by ICP/OES analysis of acid micro wave digests.

5.3 Results

5.3.1 Metal concentrations in roots and shoots

The root observation revealed that light brown and white roots were spreading densely over
the entire depth of the rhizoboxes for Prayon and Gange. We observed that the root density and
length was decreasing in the following order: Gange > Prayon > Perfoliatum. In average the
development of the root system advanced at a rate of 10, 7 and 3 cm / week into the depth of the
rhizoboxes for Gange, Prayon and Perfoliatum respectively.
Few differences were observed between the plants in root metal concentration (Table 1). Zinc and Cd concentrations were significantly higher in Gange roots. Magnesium, Al and Mn concentrations were significantly higher in Prayon roots. The accumulation of the Zn and Cd in
the shoots decreased in the order Gange >> Prayon >> Perfoliatum. The accumulation of major cations K, Ca and Mg in the shoots followed the order: Perfoliatum > Gange > Prayon.

Table 5.1: Shoot and root metal concentrations (means ± standard errors). In each column, different letter indicates significant differences at $p < 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>Cu mmol kg$^{-1}$</th>
<th>Zn mmol kg$^{-1}$</th>
<th>Cd mmol kg$^{-1}$</th>
<th>Pb mmol kg$^{-1}$</th>
<th>Mg mmol kg$^{-1}$</th>
<th>Al mmol kg$^{-1}$</th>
<th>K mmol kg$^{-1}$</th>
<th>Ca mmol kg$^{-1}$</th>
<th>Mn mmol kg$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gange</td>
<td>0.12 ± 0.02a</td>
<td>258 ± 49c</td>
<td>2.69 ± 0.40b</td>
<td>0.01 ± &lt;0.01a</td>
<td>90.2 ± 13.3b</td>
<td>9.50 ± 3.46a</td>
<td>710 ± 27b</td>
<td>315 ± 47b</td>
<td>4.46 ± 0.17b</td>
</tr>
<tr>
<td>Prayon</td>
<td>0.09 ± 0.02a</td>
<td>86.1 ± 36.4b</td>
<td>0.02 ± &lt;0.01a</td>
<td>0.01 ± 0.01a</td>
<td>32.2 ± 3.36a</td>
<td>9.17 ± 5.81a</td>
<td>424 ± 94a</td>
<td>281 ± 25a</td>
<td>0.46 ± 0.14a</td>
</tr>
<tr>
<td>Perfoliatum</td>
<td>0.25 ± 0.03b</td>
<td>4.03 ± 0.16a</td>
<td>&lt;0.01 ± &lt;0.01a</td>
<td>0.01 ± &lt;0.01a</td>
<td>225.6 ± 5.10c</td>
<td>11.6 ± 4.18a</td>
<td>1129 ± 30c</td>
<td>575 ± 29c</td>
<td>0.52 ± 0.08a</td>
</tr>
</tbody>
</table>

| Root mmol kg$^{-1}$ |                 |                  |                 |                  |                  |                 |                  |                  |                  |
| Gange | 2.93 ± 0.19a     | 32.1 ± 1.49b     | 0.46 ± 0.13b    | 1.35 ± 0.16a     | 203 ± 12.5a      | 1079 ± 153a     | 381 ± 11.9a      | 203 ± 10.0a      | 6.14 ± 0.50a     |
| Prayon| 3.54 ± 0.03b     | 20.5 ± 1.35a     | 0.03 ± <0.01a   | 1.73 ± 0.1b      | 241 ± 6.50b      | 1492 ± 5.39b    | 363 ± 12.2a      | 258 ± 6.85b      | 7.70 ± 0.20b     |
| Perfoliatum | 3.13 ± 0.55ab | 17.1 ± 1.65a | 0.03 ± <0.01a | 1.40 ± 0.20a | 211 ± 8.04a | 1050 ± 85.7a | 430 ± 53.6b | 340 ± 44.7c | 5.95 ± 0.50a |

Table 5.2: $pH$, anions concentrations DOC, oxalate and specific absorbance (means ± standard errors) in the rhizosphere solution of Thlaspi plants and in the plant-free soil (PFS) in week after sowing (WAS) 21. In each column, different letter indicates significant differences at $p<0.05$.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Nitrate (μM)</th>
<th>Sulfate (μM)</th>
<th>Phosphate (μM)</th>
<th>DOC (mM)</th>
<th>Oxalate (μM)</th>
<th>Specific absorbance $\varepsilon$ (254nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gange</td>
<td>6.7 ± 0.05a</td>
<td>13.0 ± 1.9a</td>
<td>27.7 ± 8.23a</td>
<td>120.3 ± 13.9c</td>
<td>1.92 ± 0.17b</td>
<td>2.86 ± 0.27c</td>
<td>696 ± 60.9b</td>
</tr>
<tr>
<td>Prayon</td>
<td>6.7 ± 0.08a</td>
<td>57.2 ± 48.4ab</td>
<td>77.4 ± 20.3b</td>
<td>93.6 ± 8.1b</td>
<td>1.33 ± 0.21a</td>
<td>2.06 ± 0.23b</td>
<td>684 ± 107.1b</td>
</tr>
<tr>
<td>Perfoliatum</td>
<td>6.7 ± 0.10a</td>
<td>96.3 ± 61.2b</td>
<td>50.4 ± 9.4b</td>
<td>92.5 ± 8.9b</td>
<td>2.02 ± 0.11b</td>
<td>2.59 ± 0.27b</td>
<td>396 ± 21.9a</td>
</tr>
<tr>
<td>PFS</td>
<td>6.5 ± 0.09a</td>
<td>2718 ± 270c</td>
<td>247 ± 22.9c</td>
<td>45.6 ± 2.9a</td>
<td>1.89 ± 0.08b</td>
<td>1.62 ± 0.10a</td>
<td>372 ± 15.1a</td>
</tr>
</tbody>
</table>
5.3.2 pH, inorganic anions, DOC, oxalate concentrations and specific absorbance in rhizosphere and plant-free soil

The solution pH showed little variation between the rhizospheres and the PFS (Table 5.2).

Nitrate and sulfate were significantly higher in the plant-free soil compared to any of the rhizosphere solutions (Table 5.2). The concentration of phosphate was higher in the rhizosphere of the plants than in the PFS (Table 5.2). It was highest in the rhizosphere solution of Gange.

DOC concentrations were significantly lower in the rhizosphere of Prayon compared to the other rhizospheres and the PFS. LMWOAA were mostly absent in the rhizosphere and PFS and Table 5.2 shows results only for oxalate. Oxalate concentrations were significantly higher in the rhizospheres compared to the PFS. Specific absorbance at 254 nm was higher in the rhizospheres of Gange and Prayon compared to Perfoliatum and the PFS.

5.3.3 Total metal concentrations in solutions

Calcium, Mg and K were in higher concentrations in the PFS treatment. Al was significantly lower in the rhizosphere solution of Prayon and in the PFS while Fe was significantly lower in the solution of the PFS (Table 5.3). There was a tendency for Fe to be higher in the rhizosphere of Gange (Table 5.3).

<table>
<thead>
<tr>
<th></th>
<th>Ca</th>
<th>Mg</th>
<th>K</th>
<th>Al</th>
<th>Mn</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gange</td>
<td>137 ± 10.2a</td>
<td>40.3 ± 2.5a</td>
<td>151 ± 11.0b</td>
<td>10.6 ± 0.7b</td>
<td>0.1 ± 0.03a</td>
<td>10.8 ± 4.3b</td>
</tr>
<tr>
<td>Prayon</td>
<td>136 ± 15.4a</td>
<td>46.4 ± 6.1a</td>
<td>113 ± 5.4a</td>
<td>6.3 ± 0.7a</td>
<td>1.8 ± 2.6a</td>
<td>6.1 ± 0.6b</td>
</tr>
<tr>
<td>Perfoliatum</td>
<td>134 ± 6.3a</td>
<td>46.3 ± 2.2a</td>
<td>161 ± 8.4b</td>
<td>12.0 ± 2.0b</td>
<td>0.3 ± 0.1a</td>
<td>7.2 ± 0.7b</td>
</tr>
<tr>
<td>Plant-free</td>
<td>408 ± 23.5b</td>
<td>147.5 ± 10.4b</td>
<td>211 ± 7.2c</td>
<td>7.5 ± 1.7a</td>
<td>0.1 ± 0.04a</td>
<td>2.6 ± 0.1a</td>
</tr>
</tbody>
</table>

Table 5.3: Total metal concentrations (μM) (means ± standard errors) in the rhizosphere solution of Thlaspi plants and in the plant-free soil in week after sowing (WAS) 21. In each column, different letter indicates significant differences at p < 0.05.
5.3.4 Time evolution of total and % dynamic Cu, Zn, Cd and Pb in the rhizosphere and plant-free soil solutions

Total Cu concentrations (Figure 5.1) were constant in the rhizosphere solutions while it decreased but not significantly in the PFS. Total Pb concentration decreased over time in all treatments. For Cu and Pb the total concentrations were higher for Gange and Perfoliatum. Total Zn concentration increased in the rhizosphere solution of Gange (not significantly) and Prayon over time and decreased in the rhizosphere of Perfoliatum (not significantly) (Figure 5.1). The total Cd concentration did not change over time in the rhizosphere solution of Gange; it increased in the rhizosphere solution of Prayon but not significantly and decreased but not significantly in the rhizosphere solution of Perfoliatum (Figure 5.1).

![Graphs showing time evolution of heavy metals](image)

**Figure 5.1**: Time evolution of total (nM) heavy metals in the rhizosphere solution of Thlaspi caerulescens Gange and Prayon, Thlaspi Perfoliatum and in the plant-free soil solution (PFS).

The % dynamic Cu and Pb did not change over time in any of the treatments (Figure 5.2). The % dynamic Cu and Pb were lower for Gange and Perfoliatum. The % dynamic Zn decreased
significantly in the rhizosphere of Gange and Prayon and did not change in the rhizosphere solution of Perfoliatum (Figure 5.2). At the end of the experiment, the % dynamic Zn was significantly lower in the rhizosphere of Gange and Prayon than in that of *T. perfoliatum* and the plant-free soil (Figure 5.2). The % dynamic Cd decreased significantly in the rhizosphere of Gange and did not change in the rhizosphere of Prayon and Perfoliatum (Figure 5.2). Also the complexation of Cd was much stronger in the rhizosphere of Gange compared to the other rhizospheres and the PFS.

**Figure 5.2:** *Time evolution of % dynamic heavy metals in the rhizosphere solution of Thlaspi caerulescens Gange and Prayon, Thlaspi Perfoliatum and in the plant-free soil solution (PFS).*

### 5.3.5 pH dependence of the dissociation of the metal complexes

Figure 5.3 shows the dependence of the ratio between dynamic and total metal concentrations on the values of pH.
No treatments effect was observed on complexation curves of Cu and Pb (Figure 3). The differences between the four curves were small. At very low pH (2-3) the complexation of Cu in the rhizosphere of all plants was lower compared to the PFS and the Gange desorption curve seem to be even slightly lower at pH 2.5.

![Graph showing complexation of Cu, Zn, Cd, and Pb](image)

**Figure 5.3:** The effect of pH on the complexation of the Cu, Zn, Cd and Pb in the rhizosphere solution of Thlaspi plants and in plant-free soil (PFS).

At pH values observed in soil solution (between 6-7) during the experiment, there was a great difference in the degree of complexation between the rhizosphere solutions and the PFS for Cd and to a lesser extent for Zn. The shape of the curves for Zn was similar between the rhizosphere and the PFS solutions but at higher pH we observed a shift for *T. perfoliatum* and the PFS solutions meaning that the complexation strongly decreased for these two examples.
Cd was obviously much more complexed in the rhizosphere solution of Gange. The complexation curves for Cd in the rhizosphere of Gange exhibited two steps: a first part of the Cd was released at pH around 5.5 but part of the Cd was strongly bond and was released only when the solution was acidified to pH 2.5. Cd was almost completely free at pH 7 in the PFS.

In the rhizospheres and PFS the model describe well the pH dependence of Cu and Pb. For Zn it gave a quite good description of the PFS, although the higher complexation strength of Gange and Prayon on Perfoliatum was not described. The calculated complexation for a PFS for Cd seems to be too low whereas it is too strong in for the rhizosphere solutions.

5.4 Discussion

5.4.1 Metal concentrations in roots and shoots

The uptake of Zn and Cd by *Thlaspi caerulescens* Gange was really high, in agreement with the finding of Hammer and Keller (2002) who used the same soil. This confirmed the extraordinary ability of the plant to accumulate these metals in the shoot. It is also well known that *Thlaspi caerulescens* Prayon ecotype possess a limited accumulation capacity for Cd (Lombi et al. 2001) which was also demonstrated in this study with an accumulation of Cd in the shoot being more than hundred time less compared to Gange ecotype. This may be explained by the absence of highly selective Cd transport system in the root cell membranes compared to the Gange ecotype. The fact that Prayon accumulated about three times less Zn in its shoot than the Gange ecotype was not expected. Zhao et al. (2002) found a difference of 1.5 fold in the Zn uptake between the two ecotypes in a hydroponic study. *Thlaspi perfoliatum* accumulated about thousand times less Cd and a hundred times less Zn in its shoot, and thus as expected did not show any hyperaccumulating traits.

5.4.2 Total metal concentrations

*Thlaspi* is a member of the Brassicaceae family which is well known for its high demand in sulphur which can explain the lower sulfate concentration in the rhizosphere compared to the PFS. Nitrate, Ca, Mg and K being major nutrients were also found to be lower in the rhizospheres compared to the PFS. Differences between PFS and rhizosphere solutions of planted soil.
indicated that phosphate was mobilized by roots and that with time the ratio between depletion by uptake and replenishment by mobilization increased. *T. caerulescens* is known to be very efficient in extracting P from soils. Even when available P content is low, this species rarely needs P fertilization (Zhao et al. 1998). As in the case of phosphate there was mobilization of iron in the rhizosphere solution of all three plants than in the PFS. These differences in total metal concentrations in each rhizosphere and in the PFS can definitely influence heavy metal complexation in solution.

The Zn was the only metal that showed an increase in total concentration with time in the rhizosphere solution of Prayon and Gange, but not in the other treatments (*T. perfoliatum* and PFS) indicating a slight mobilization of this metal in the rhizosphere of *Thlaspi caerulescens*. The total concentration of Zn did not differ to a great extent between the two *Thlaspi caerulescens* and the PFS at the end of the experiment.

For Cu and Pb which are not accumulated by the *Thlaspi* plants the concentrations were higher in the rhizosphere of Gange and Perfoliatum compared to the PFS. The lower value for Prayon does not seem to suit into this picture. However, we have to consider the significantly lower DOC concentration in the Prayon rhizosphere. Cu and Pb concentrations in soil solution are mainly controlled by the complexation with DOC (Stevenson 1994). The lower concentration of Cu and Pb observed in PFS despite similar DOC may be due to different properties of DOC in the PFS compared to the rhizosphere of the plants.

### 5.4.3 Metal speciation

The % dynamic fraction of Zn and Cd decreased considerably over time in the rhizosphere solution of Gange and Prayon and there was a great difference compared to *Thlaspi perfoliatum* and the PFS at the end of the experiment. These results may indicate that it is not primarily mobilization that is involved in the hyperaccumulation mechanism of Zn and Cd by *Thlaspi caerulescens* but that the complexation of those metals is influenced by the plants.

The active metal uptake by a hyperaccumulator can only be efficient if the replenishment of the soluble phase is fast enough to compensate for the removal by the active uptake. On one hand it is well known that the sorption-desorption processes of metals from soil might be kinetically limited (Filius et al. 1998; Hinz and Selim 1994; Selim et al. 1992). On the other hand the active
uptake of metals by *Thlaspi caerulescens* has already been demonstrated ([Lombi et al. 2001; Pence et al. 2000] and recently [Ingwersen et al. 2006]) suggested that *Thlaspi caerulescens* can accelerate the replenishment of the depleted rhizosphere solution. Our study suggest that increase complexation of Zn and Cd in the rhizosphere of *Thlaspi caerulescens* reduces the dynamic concentration of the metal in solution and thus increases the driving force for metal mobilization, thus accelerating the replenishment of the soil solution. The hyperaccumulation of Zn and Cd by *Thlaspi caerulescens* seems to be supported by a strong complexation of Zn and Cd.

### 5.4.4 Origin of the complexing ligands in the rhizosphere

The potential ligands in the rhizosphere of Ganges and Prayon appear to be selective for Cd and Zn as they do not change Cu and Pb speciation. These characteristics point towards a specialized ligand selective for Zn and Cd. DOC for example shows complexation increasing in the order Cd < Zn < Pb < Cu ([Stevenson 1994](#)).

Results from our study confirm the findings by several authors ([Knight et al. 1997; McGrath et al. 1997; Zhao et al. 2001]) that *Thlaspi caerulescens* does not change the solution pH or exude high amounts of LMWOAA. Root exudates have been found to results in a complex network of reactions in the rhizosphere solution that as an example led to the mobilization of DOC, major cations (e.g. Ca) and metals (e.g. Fe, Al, Zn, Cu), and although metals such as Cu, Zn, Cd and Pb only make up a very small fraction of the citrate complexes, these metals are strongly influenced by exudation of LMWOAA such as citrate (Chapter 4). On the other hand it cannot be ruled out that other compound than the one analyzed are involved in the rhizosphere of *Thlaspi caerulescens*.

The baseline of UV/VIS absorbance represents dissolved organic matter deriving from soil organic matter degradation. The change in relative absorptivity can then be exudation of UV absorbing compounds or an indirect effect of the plant on the soil organic matter degradation. The specific absorbance at 254 nm which was higher in the rhizospheres of hyperaccumulators is mainly related to the aromaticity, hydrophobic content and characterization of humic substances ([Abbt-Braun and Frimmel 1999; Artinger et al. 2000; Croué et al. 2003; Hur and Schlautman 2003; Kalbitz et al. 2000]). Thus although UV spectroscopic analyses of DOC do not provide specific information about individual molecules, we may conclude that the rhizosphere solution
of Gange and Prayon were characterized by DOC with higher hydrophobic and aromatic properties compared to Perfoliatum and PFS. More hydrophobic compounds such as certain phenols and also mucilage and mucigel may be involved in the complexation of Zn and Cd in the rhizosphere solution of *Thlaspi caerulescens*. From the results shown in Table 2 it seems that DOC in the rhizosphere solution of all plants was characterized by a material of lower molecular weight compared to the PFS.

The results from the modeled desorption curves at least for Cu, Zn, and Pb suit well to the hypothesis that the DOC in the rhizosphere was different compared to PFS. For Cd there seems to be a problem that the binding by the DOC in this soil was extremely low.

**Acknowledgements**

This work was supported by project C02.0084, State Secretariat for Research and Education, Switzerland, within COST Action 631 (Understanding and Modelling Plant-Soil Interactions In the Rhizosphere Environment) and the Fond Québécois de la Recherche sur la Nature et les Technologies. The rhizoboxes were designed and produced by Arthur Kölliker, Swiss Federal Institute for Forest, Snow, and Landscape Research (WSL).
Chapter 5 Zn and Cd in the rhizosphere of Thlaspi caerulescens

References


Chapter 5 Zn and Cd in the rhizosphere of Thlaspi caerulescens


Keizer, M.G., VanRiemsdijk, W.H. 2002 A computer program for the equilibrium calculation of speciation and transport in soil-water systems (ECOSAT 4.7), Department of soil quality, Wageningen University.


Lindsay, W.L. 1979 Chemical equilibria in soils, Wiley Interscience, New York.


6 Conclusions

The main goals of this study were 1) to test a modification of the classical rhizobox micro suction cup system design to obtain a better precision of the localized in-situ sampling of soil solution and, in addition, to minimize the risk of biodegradation in the samples 2) to investigate in-situ the temporal and spatial patterns of organic anions exuded by cluster roots of *Lupinus albus* and their relation to soil solution chemistry, in particular to nutrients such as phosphate, nitrate and sulfate 3) to investigate the impact of the exudation of organic anions by *Lupinus albus* on the concentration and speciation of macro and micronutrients in different rhizosphere and soil solution samples 4) to investigate the heavy metal solubility and complexation in the rhizosphere solution of *Thlaspi caerulescens*.

6.1 Modified micro suction cup/ rhizobox approach for the in-situ detection of organic acids in rhizosphere soil solution

In the present study we first investigated a modification of the classical rhizobox/micro suction cup system to make it suitable for the collection and analysis of organic anions in the rhizosphere. In order to show the potential of the method, we tested the modified system with *Lupinus albus* as a model plant known to exude large amounts of citrate. We demonstrated that with our modified rhizobox micro suction cup system we were able to detect in-situ organic acids exuded from roots and to follow temporally an exudation process and its effect on soil solution chemistry. With this system we could also detect differences between bulk soil, rhizosphere of cluster roots and rhizosphere of other roots (e.g., nodules). Moreover, to our best knowledge, this is the first time the exudation of organic acids by *Lupinus albus* has been shown in-situ in soil solution. Based on our results, the system is a good choice to study strong root exudation by single roots in-situ.
6.2 Organic acid exudation and nutrient anions in the rhizosphere soil solution of *Lupinus albus*

The temporal patterns and spatial extent of organic anion exudation into the rhizosphere solution of *Lupinus albus*, and its relation with the nutrient phosphate, nitrate and sulfate was investigated by means of the new rhizobox micro suction cup method. We compared the soil solution in the rhizosphere of cluster roots with that in the vicinity of normal roots, nodules and bulk soil. From our comprehensive in-situ soil solution study on citrate exudation by *Lupinus albus* cluster roots we conclude the following. The exudation of citrate follows a diurnal pattern with higher rates during the afternoon. The exudation burst can occur in several waves during the lifetime of a cluster root. Exuded organic acids can be transported up to 5mm from the cluster root apex. Since cluster roots are not only characterized by high organic acid exudation but are also hot spots of phosphate uptake, mobilization of phosphate by citrate exudation is difficult to assess in-situ. Nevertheless, some relations between the temporal patterns of phosphate and citrate concentrations during the lifetime of a cluster roots could be shown. Furthermore, our study demonstrated again the potential of the rhizobox micro suction cup technique to study the influence of individual roots on soil solution chemistry. In particular, this method seems to be well suited for a more detailed future study on rhizosphere effects around root nodules.

6.3 Metal solubility and speciation in the rhizosphere of *Lupinus albus*

The influence of root exudation on nutrient and metal species in the rhizosphere of *Lupinus albus* was investigated. This study demonstrated that the exudation of citrate by *Lupinus albus* resulted in a complex network of reactions that led to the mobilization of DOC, major cations (e.g. Ca) and metals (e.g. Fe, Al, Zn, Cu). Although the metals Cu, Zn, Cd and Pb only make up a very small fraction of the citrate complexes, these metals are strongly influenced by exudation. The effect of exudation on metal solubility in the rhizosphere is twofold: on the one hand metals like Zn, Fe and Al are directly mobilized and complexed by citrate. The speciation calculations show that for these metals a significant fraction is bound to citrate during the exudation event. On
91 Chapter 6 Conclusions

the other hand there is also an indirect effect of citrate through mobilization of DOC which in turn is then complexing and mobilizing metals such as Cu and Pb.

6.4 Mobilization and complexation of Zn and Cd in the rhizosphere of Thlaspi caerulescens

The mobilization and complexation of metals in the rhizosphere of different Thlaspi plants, and its change over time was investigated. The results from this in situ rhizobox study may indicate that it is not primarily mobilization that is involved in the hyperaccumulation mechanism of Zn and Cd by Thlaspi caerulescens but that the complexation of those metals is influenced by the plants. The potential ligands in the rhizosphere of Ganges and Prayon are selective for Cd and Zn and do not change Cu and Pb speciation. UV absorbance spectroscopy results revealed that the rhizosphere solutions of Thlaspi caerulescens Gange and Prayon were characterized by higher specific absorbance compared to Thlaspi perfoliatum and the bulk solution.

6.5 Outlook and open questions

The results from this study show that the use of the modified rhizobox micro suction cup system is well suited for the in situ investigation of rhizosphere processes occurring throughout the plant growth.

The fact that we observed concentrations highly variable and up to 15 mM of citrate in cluster root rhizospheres under P sufficient soil conditions need further investigation and how these variabilities and concentrations would have been under P stress conditions. Also the variability of exudation between different cluster roots of one single lupin plant has not received much attention so far. In addition the mobilization of phosphate by the exuded citrate was difficult to assess in-situ.

To our knowledge no studies have been done characterizing the soil solution composition surrounding nodules. The reason that we have measured high acetate concentrations in the rhizosphere solution of the nodule remains unclear. Whatever the reason for the difference in acetate, the rhizosphere samples at low and high acetate concentration clearly show differences in
total metal solubility and metal speciation. These results warrant a further systematic investigation of this very active rhizosphere compartment.

The strong influence of root exudates such as citrate on metals mobilization have been demonstrated in this study. However environmental impacts such as metal leaching during exudation events warrant further investigation.

The role of the complexation of Zn and Cd in the rhizosphere of *Thlaspi caerulescens* have been demonstrated in this work as an important hyperaccumulation mechanism. However further research are needed in order to identify the ligand responsible for the complexation of those two metals.
Acknowledgements

I would like to thank Prof. Rainer Schulin, Dr. Bernd Nowack, Dr. Jörg Luster and Marie-Louise Tercier for their advice, help and guidance.

I would like also to thank my family, friends and colleagues for their help and support which enable me to carry out and finish this work. I would especially like to thank Arthur Kölliker who designed and produced the rhizoboxes, Marlies Petzold and Lena Püschel who helped with the set-up of the rhizoboxes and the intensive sampling of soil solutions and Ursula Graf from central laboratory WSL. I would like also to thank Dr. Martin Schroth for his essential help with the ion chromatograph and François Bujard from the University of Geneva for the construction of the voltammetric analysis cell. Finally, I would like to thank Björn Studer, Anna Gründwald and René Saladin for their technical help in the laboratory and Iso Christl for his technical help and assistance with the modeling.

Lastly I would like to thank the Fond Québécois de la Nature sur la Recherche et les Technologies for giving me the opportunity to carry out my PhD thesis in Switzerland, and the other funder of this work, the State Secretariat for Research and Education, Switzerland, within COST Action 631 (Understanding and Modeling Plant-Soil Interactions In the Rhizosphere Environment).
Curriculum Vitae

Surname                          Dessureault-Rompré
First Name                       Jacynthe
Date of Birth                    14.03.1977
Place of Birth                   Grand-Mère, Québec, Canada
Nationality                      Canadian

School Education
1994-96                          Collège de Trois-Rivières, Qc, Ca.

Higher Education
2000-2002                        MSc. Soil and Environmental Science, Laval University, Qc, Ca.

Occupation
Sept 2002 - Sept 2003            Assistant for the Agriculture and Environment service of “IAG”
                                  (Institut d’Agriculture de Grangeneuve), Fribourg, Switzerland.
Sept 2003 - present              PhD Studies, Soil Protection Group, Institute of Terrestrial
                                  Ecosystems, Swiss Federal Institute of Technology (ETH)
                                  Zurich, Switzerland