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How White Lupin Can Survive In Soils With Sparingly Available Phosphate: Cluster Root Secretion Physiology And Plant microbe Interactions

Thesis presented to the Faculty of Science of the University of Neuchatel
for the degree of Doctor of Science

By Laure Weisskopf

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IMPRIMATUR POUR LA THESE

How white lupin can survive in soils with sparingly available phosphate: cluster root excretion physiology and plant-microbe interactions

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Neuchâtel, le 5 juillet 2005

La doyenne:

Prof. M. Rahier

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Summary

In response to soils with low available phosphate, plants have evolved different strategies. One of them is the formation of special root structures called proteoid roots or cluster roots. Cluster roots are almost ubiquitous in the Australian Proteaceae family and they are also found as exceptions in other plant species. Such an exception is white lupin, *Lupinus albus* L., our model plant. This annual leguminous plant is the sole cluster root forming species of agricultural importance. White lupin cluster roots secrete large amounts of organic acids, mostly citrate and malate, and concomitantly acidify the rhizosphere. Organic acid secretion and rhizosphere acidification take place at a particular stage of cluster root development, which is called the "mature" stage. In the present work, we investigated the secretion physiology of different developmental stages of white lupin cluster roots and their impact on the rhizosphere microflora. Isoflavonoid contents and secretion patterns were characterized in growing cluster roots of white lupin. Genistein and hydroxygenistein, as well as their glycosylated conjugates, were the major isoflavonoids identified. While internal contents remained stable during cluster root development, the amounts secreted varied in function of cluster root stage. Highest secretion was observed at the juvenile and immature stages of cluster roots. The secretion of isoflavonoids by white lupin seedlings was strongly modified when elicited with the presence of bacteria or fungi and the response was strain-specific.

The impact of the secretion activity of white lupin cluster on the rhizosphere microbial communities was investigated as well. Our results showed that the bacterial communities were influenced by the secretion activity of cluster roots: a reduced abundance (cultivable and total) of bacteria, as well as a decreased richness

(DGGE profiles) characterized the mature stage of cluster roots. Approximately 50 % of the bacterial populations isolated from the mature stage were able to grow in a low pH (4) medium, while only 20 % of the bacteria isolated from juvenile cluster roots could do the same, suggesting that the pH decrease occurring transiently in the rhizosphere of mature cluster roots could be the cause of the decreased abundance of bacteria observed at this stage. In contrast, no inhibitory effect of the secreted isoflavonoids could be evidenced *in vitro* on bacteria. Only fungi showed a reaction to these compounds, namely a stimulated sporulation. PGPR (plant growth promoting rhizobacteria) activities were also assessed in strains isolated from white lupin cluster roots and the results suggested that auxin producing bacteria could be involved in cluster root formation. Moreover, associative nitrogen fixation was analyzed by fingerprinting techniques on the *nifH* pools amplified from DNA and RNA samples from different cluster root stages. No amplification was observed for mature cluster roots, while amplification from extracted DNA, and in one case also from extracted RNA, was obtained for juvenile and senescent cluster roots. This indicates that associate nitrogen fixation might also take place in the rhizosphere of cluster roots, in addition to the symbiotic fixation in nodules forming on non cluster roots.

Phosphorus is a major limiting factor for agricultural yields in many regions of the world and white lupin may be a promising future crop in this respect, since it is able to acquire both phosphate through cluster root formation and nitrogen through symbiotic fixation. The better understanding of cluster root functioning and of the potential plant microbe interactions in their rhizosphere, which has been achieved through this work, hopefully will contribute to a successful transfer of knowledge from model systems to real field conditions.

Résumé

Au cours de l'évolution, les plantes ont développé différentes stratégies pour survivre sur les sols où le phosphate est peu disponible. L'une d'entre elles est la formation de structures racinaires particulières, que l'on appelle racines protéoïdes ou « cluster roots ». Presque toutes les plantes de la famille australienne des Protéacées développent ces racines, alors qu'on les observe seulement à titre d'exception chez les autres espèces. Le lupin blanc, *Lupinus albus* L., notre plante modèle, est une telle exception. Cette légumineuse annuelle est la seule espèce d'importance agronomique qui forme des racines protéoïdes. Les racines protéoïdes du lupin blanc sécrètent de grandes quantités d'acides organiques, surtout du citrate et du malate, et provoquent simultanément une acidification de la rhizosphère. Cette sécrétion et acidification se déroulent à un stade bien particulier du développement des racines protéoïdes, le stade « mature ». Dans le travail qui suit, nous avons étudié la sécrétion racinaire à différents stades de développement des racines protéoïdes du lupin blanc ainsi que l'influence de cette sécrétion racinaire sur la microflore rhizosphérique. Nous avons caractérisé les isoflavonoïdes produits et excrétés dans les racines protéoïdes en croissance. Les isoflavonoïdes les plus importants étaient la génistéine et l'hydroxygénistéine, ainsi que leurs conjugués glycosylés. Alors qu'aucun changement n'a pu être observé dans les contenus internes d'isoflavonoïdes durant le développement des racines protéoïdes, la sécrétion de ces composés, elle, variait en fonction du stade de développement, avec la plus forte sécrétion dans les jeunes racines et les racines prématures. Une modification importante de cette sécrétion d'isoflavonoïdes par les jeunes pousses de lupins a été observée lorsque les graines avaient germé en présence de bactéries ou de champignons. Cette réaction à la présence de micro-organismes lors de la germination était spécifique à la souche de bactérie ou de champignon utilisée.

Nous avons également investigué l'impact de la sécrétion des racines protéoïdes du lupin sur les communautés microbiennes de la rhizosphère. Nos résultats ont montré que les communautés bactériennes étaient influencées par l'activité de sécrétion des racines protéoïdes : le stade mature des racines protéoïdes était caractérisé par une

plus faible abondance de bactéries (cultivables et totales), ainsi qu'une moins grande richesse (profils DGGE). Environ 50 % des populations bactériennes isolées du stade mature des racines protéoïdes était capables de pousser sur des milieux à bas pH (4), alors que seuls 20 % des populations isolées du stade juvénile pouvaient faire de même, ce qui suggère que la baisse de pH qui se produit de manière temporaire dans la rhizosphère des racines protéoïdes matures pourrait être la cause de la baisse d'abondance bactérienne observée précisément à ce stade. En revanche, aucun effet inhibiteur des isoflavonoïdes excrétés par le lupin blanc n'a pu être observé *in vitro* sur la croissance des bactéries. Ces isoflavonoïdes ont provoqué une réaction uniquement chez certains champignons, dont la sporulation a été stimulée. Des activités de type PGPR (plant growth promoting rhizobacteria) ont aussi été testées sur les souches isolées de la rhizosphère des racines protéoïdes du lupin blanc et les résultats ont suggéré que la production d'auxine par les bactéries pourrait être impliquée dans la formation des racines protéoïdes. De plus, la fixation associative d'azote a été analysée par des méthodes de « fingerprinting » sur la base de fragments du gène *nifH* amplifié à partir des ADN et des ARN extraits de différents stades des racines protéoïdes. Aucune amplification n'a été observée pour les racines protéoïdes matures, alors que l'amplification a été possible pour les juvéniles et les sénescentes à partir d'ADN et également à partir d'ARN dans un cas, ce qui laisse penser que la fixation associative d'azote pourrait avoir lieu dans la rhizosphère des racines protéoïdes, en plus de la fixation symbiotique dans les nodules, qui se forment en général sur les racines non protéoïdes.

Le phosphore est un des éléments majeurs qui limitent les rendements de l'agriculture dans bien des régions du monde et le lupin blanc pourrait être une plante prometteuse à cet égard, puisqu'il est capable à la fois d'acquérir le phosphate en produisant des racines protéoïdes et de fixer l'azote en association avec des bactéries symbiotiques. A l'issue de ce travail, nous avons acquis une compréhension plus approfondie du fonctionnement des racines protéoïdes et des interactions entre la plante et les micro-organismes de la rhizosphère. Ces connaissances pourront, je l'espère, être transférées du laboratoire à la situation concrète de la culture au champ.

CHAPTER I

General introduction

Chapter I: overview

1.1. PLANT NUTRITION UNDER PHOSPHORUS DEFICIENCY

In addition to the carbon dioxide and oxygen uptake through the stomata at the shoot level, plants rely on water and mineral nutriments from the soil for their growth and development. In the temperate regions, plant production of biomass very often is limited by nutrient deficiencies, despite the addition of fertilizers. For instance, phosphorus (P) deficiency is reducing crop yield on more than 30 % of the world's arable land (Vance et al., 2003). This is linked to the bioavailability of nutrients: some elements, like phosphorus or iron, may be present in high quantities in soils, but in precipitated forms, which are not available to the plants. It has been reported that plants in general only take advantage of less than half of the quantity of fertilizers applied, while the remaining leaches into groundwater. In addition to the negative ecological impact of this excessive fertilizer application, such an extensive use of fertilizers is likely to be restrained in the near future, since mineral elements are not an inexhaustible resource. It has been estimated that inexpensive sources of rock phosphate could be depleted in as little as 60 to 80 years (Vance, 2001). For these reasons, it is of central interest to better understand the mechanisms of plant nutrient acquisition, in order to deal with natural resources in a reasonable and sustainable way.

Mineral ions are transferred through passive diffusion mechanisms from the soil solution to the root, following the concentration gradient. The uptake at the root level acts like a sink and drives the nutrient flow from the soil to the root. However, plants do not have direct access to the mineral ions which are not in the soil solution, but which are present in an insoluble state, in complex with metals for example. These precipitated and not accessible mineral species thus first have to be solubilized before they can be taken up by plants.

Successful solubilization mechanisms will depend on the mineral ion considered, on its predominant chemical form and on the soil type. Since this work focuses on phosphorus deficiency, we will concentrate here on phosphate uptake. Figure 1 (Schachtman et al., 1998) summarizes the main forms of phosphorus in soil and the ways of enhancing P

uptake. Ca-phosphate (Ca-P) complexes will preferentially form in calcareous soils, while Fe-Al-phosphates (Fe-Al-P) will be more abundant in acidic soils. Secretion of carboxylates and acidification of the root environment are two strategies to improve phosphate availability which are commonly found among plants (Imas et al., 1997; Kirk et al., 1999; Zhang et al., 1997). While acidification helps desorbing phosphate from Ca-P complexes, carboxylates acts as chelators and ligand exchangers for the solubilization of Fe-Al-P complexes (Gerke et al., 1994).

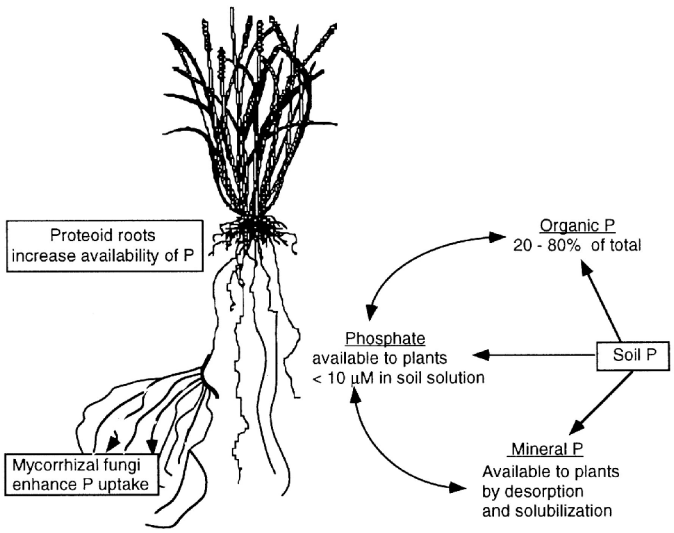

Figure 1 : P acquisition strategies (from Schachtman et al.***, 1998)***

In addition to the solubilization of phosphate (Pi) from inorganic metal complexes, plants also can take up phosphate from organic phosphate pools. This occurs through the secretion of extracellular phosphatases and phytases (Gaume et al., 2001a; Richardson, 2001), hydrolytic enzymes which cleave phosphate ions from organic molecules. Specific transporters with a high affinity for Pi are also produced in higher quantities when the plant faces phosphate deficiency (Liu et al., 2001).

Moreover, about 80 % of the plant species overcome the problems related to P deficiency by establishing a symbiotic association with mycorrhizal fungi, which provide the plant

with mineral nutriments, especially phosphate and nitrogen, through their high affinity uptake mechanisms and their large volume of soil explored.

Finally, among the 20 % of non-mycorrhized plants species, some have developed a special strategy to cope with phosphate deficiency: the formation of special root structures, called cluster roots or proteoid roots (Purnell, 1960). These roots secrete high amounts of organic acids and protons and represent a very efficient alternative to the mycorrhizal association (see below). Such a cluster-rooted plant is white lupin (*Lupinus albus*), our model plant.

1.2. *LUPINUS ALBUS*: A SPECIALIST OF LOW P SOILS

1.2.1. Getting to know white lupin

White lupin, *Lupinus albus* L., is an annual species of the genus *Lupinus*, tribe *Genisteae* and family *Fabaceae*. It owes its Latin name "*Lupinus*", literally "little wolf" to the fact that it was held – wrongly – responsible for the poverty of the soils where it was growing. "albus" refers not only to the color of the flowers (Figure 2 A), but also to the fine border of white hairs which surrounds the leaves (Figure 2 B).

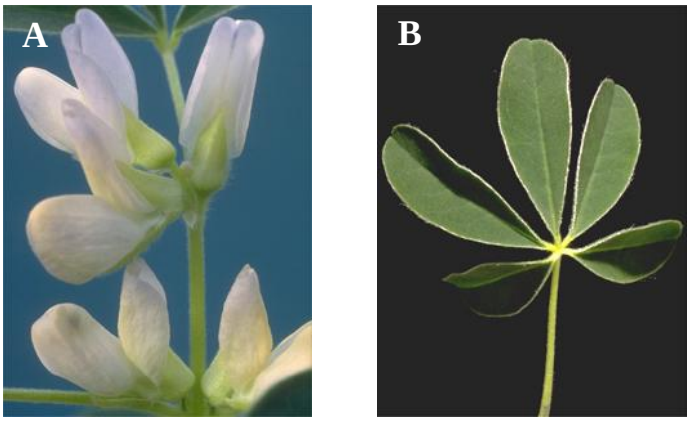

Figure 2: **Lupinus albus *L.***

Flowers (A) and leaf (B)

White lupin is an Old World species distributed along the Mediterranean (see Figure 3 for a picture of a lupin field in Italy) and the Nile Valley.

Figure 3: White lupin field

Lupin field in Piemonte (Italy)

Photo: M. Aragno

L. albus has been cultivated there for thousands of years. About 25 years ago, it was also introduced to Australia and has been cultivated more and more extensively since then (Marsh et al., 2000). White lupin is used mainly for i) human nutrition, because of the high protein and oil levels in seeds, ii) green manure, improving soil structure, as well as nitrogen and phosphorus contents in poor sandy soils and iii) green forage or seeds introduced as nutrient complements to the diet of ruminants (Huyghe, 1997). White lupin grows more easily on soils of neutral or acidic pH, but in contrast to the other cultivated *Lupinus* species, *L. angustifolius*, *L. luteus* and *L. mutabilis*, it is also able to cope with calcareous soils (Hinsinger and Gilkes, 1995).

With respect to symbiotic associations, white lupin forms nitrogen fixing nodules in symbiosis with rhizobiae of the genus *Bradyrhizobium* (González-Sama et al., 2004; Robinson et al., 2000) but it belongs to the 20 % of plant species which are not mycorrhized. Still, white lupin is well-known for its resistance to phosphate starvation and its P acquisition strategy lies in the formation of particular root structures, called proteoid roots, or cluster roots.

1.2.2. Cluster roots

Cluster roots were first observed and characterized by Purnell in 1960 as "clusters of rootlets of limited growth, which form on a lateral root". These cluster roots are a common trait in many species of the Proteaceae family and this is why they were called "proteoid roots". In the present work, we will refer to them as "cluster roots". Figure 4 shows cluster roots from different species (Shane et al., 2004; Lambers et al., 2003): from *Banksia grandis* (A), *Hakea prostrata* (B), two *Proteaceae* and from *L. albus* (C and D).

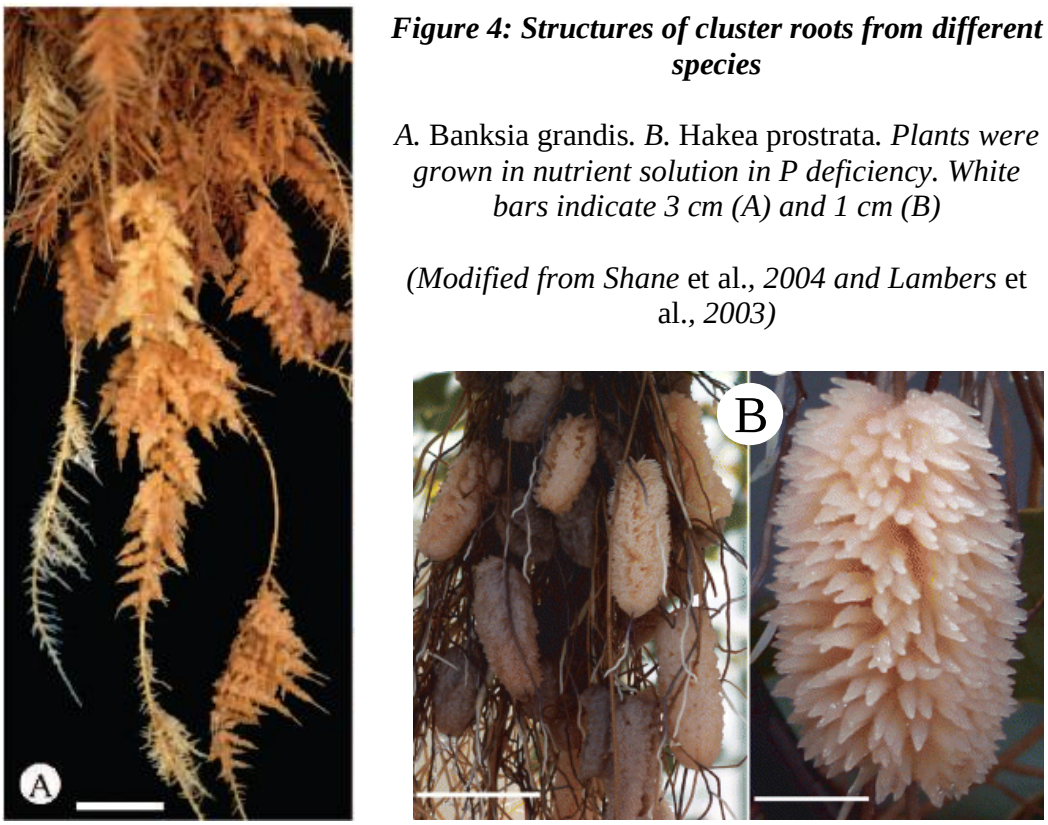

Figure 4: Structures of cluster roots from different species

A. Banksia grandis. *B.* Hakea prostrata. *Plants were grown in nutrient solution in P deficiency. White bars indicate 3 cm (A) and 1 cm (B)*

(Modified from Shane et al., *2004 and Lambers* et al., *2003)*

Figure 4 (cont.): Structures of cluster roots from different species

C. Root system of white lupin grown in hydroponic culture in the absence of phosphate. D. Zoomed view showing different growth stages of cluster roots

Picture by M. Aragno

Cluster roots are almost ubiquitous in the *Proteaceae* family and also form in some other plant families, like in the *Casuarinaceae*, *Mimosaceae*, *Fabaceae*, *Myricaceae* and *Moraceae* (Dinkelaker et al., 1995). Interestingly, a majority of these families also are characterized by the occurrence of symbiotic nitrogen fixation: members of the families *Casuarinaceae* and *Myricaceae* are known to form nodules with the bacteria belonging to the genus *Frankia*, while members of the family *Fabaceae* produce nodules in association with the *rhizobiaceae* (*Rhizobium* sp., *Bradyrhizobium* sp.). This co-occurrence of cluster roots and symbiotic nitrogen fixation in evolutively very different families probably represents more than a pure coincidence and although no study up to now assessed this question, one can assume that the modification of the root structure which characterizes both phenomena could be genetically linked. Cluster root formation, development and secretion physiology have been intensively studied in the last years and the present knowledge on the topic has been recently reviewed in three contributions (Lamont, 2003; Neumann and Martinoia, 2002; Shane and Lambers, *in press*). We will thus not enter into details about general considerations on cluster roots, but rather concentrate on the case of white lupin.

1.2.3. Cluster root formation and secretion physiology in white lupin

Cluster root formation

Many studies on cluster root formation and physiology have been conducted using white lupin as a model plant (Dinkelaker et al., 1989; Gardner et al., 1982a, 1982b, 1982c 1983; Johnson et al., 1994, 1996a, 1996b; Marschner et al., 1987; Massonneau et al., 2001; Neumann et al., 1999, 2000; Penaloza et al., 2002; Shane et al., 2003b). Cluster roots are induced by phosphorus deficiency and, in some cases, by iron deficiency. Experiments based on foliar application of P (De Vos et al., 2001; Marschner et al., 1987) revealed that the internal P status of the plant is a key factor for induction of cluster root formation. Nevertheless, it has also been observed that cluster roots develop preferentially in the upper soil layers or in other nutrient rich patches of soil (Purnell, 1960; Skene, 1998) and this indicates that external soil factors also may be involved in the control of cluster root formation. The potential role of soil microorganisms in cluster root formation has remained uncertain, some studies indicating that bacteria might be necessary for cluster root formation (Lamont and McComb, 1974; Malajczuk and Bowen, 1974) while others demonstrating the production of cluster roots also in sterile conditions (Gardner et al., 1982a in Lamont, 2003) in two experiments, whereas in the third one, the non inoculated autoclaved pots ("sterile controls") which were contaminated with "a few microbial species" showed six times fewer cluster roots than the inoculated autoclaved pots, who harbored a "diverse microflora". Clearly, as long as healthy, well-growing plants cannot be cultivated in sterile conditions, with appropriate controls as inoculated plants, the bacterial impact on cluster root formation will remain an open question.

A few studies investigated the role of hormones in cluster root development and it could be shown that auxin promotes cluster root formation, while auxin inhibitors or cytokinins play an inhibitory role (Gilbert et al., 2000; Skene and James, 2000). Moreover, Uhde-Stone et al. (2000) observed an enhanced expression of an expressed sequence tag (EST) with homology to an ACC oxidase, involved in the biosynthesis of ethylene. They showed that cluster root initiation was not affected by ethylene inhibitors and that this

hormone thus apparently does not influence directly the formation of cluster roots. Still, ethylene might favor the dense formation of root hairs, as suggested by Borch et al. (1999).

Secretion physiology and P uptake mechanisms

The phosphate solubilization mechanisms in cluster roots are summarized in Figure 5.

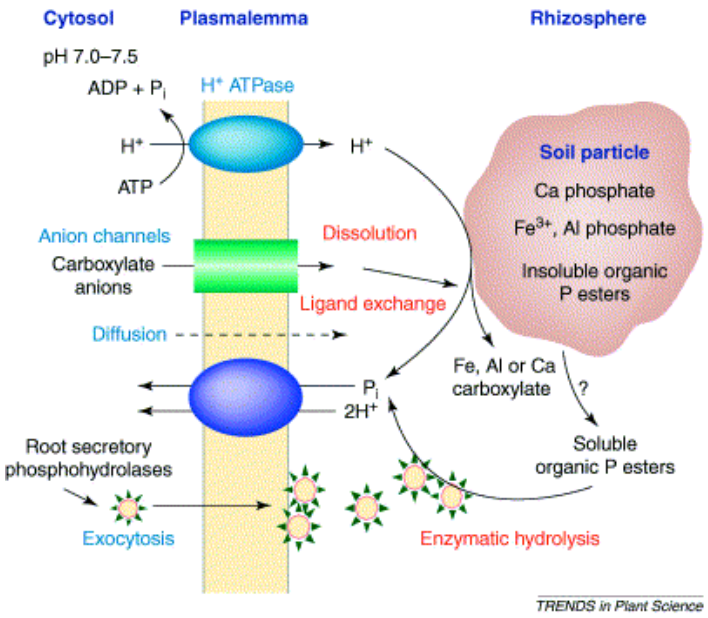

Figure 5: P uptake by cluster roots

Model for root-induced chemical phosphate mobilization in the rhizosphere by exudation of carboxylates, protons and root secretory phosphohydrolases.

(From Neumann and Martinoia, 2002)

As mentioned above, the only available source of phosphorus is the inorganic phosphate (Pi) present in the soil solution. The Pi concentration varies in function of soil type but it has been estimated (Schachtman et al., 1998) that it usually does not exceed 10 µM. Most of the inorganic phosphate is bound to Ca or Fe/Al-humic acid complexes. Solubilization of Pi from these complexes cannot be achieved solely by rhizosphere acidification; it needs the secretion of organic acids like citrate or malate. These organic anions, which are secreted with a concomitant release of protons (Sas et al., 2001), solubilize Pi by ligand exchange, taking the place of Pi in the soil matrix. Gerke et al. (1994) and Braum (1995) showed that organic anions can desorb Pi from Fe/Al complexes by chelation of

the metal cation to which Pi was bound, thus liberating Pi and suppressing its reabsorption.

In addition to this solubilization of Pi from the precipitated, inorganic forms, cluster roots also secrete hydrolytic enzymes mediating the Pi acquisition from the organic pool of phosphorus as well, which can represent up to 80 % of the total soil P, depending on the soil type. Two kinds of phosphohydrolases have been described, phosphatases on the one hand (Gilbert et al., 1999; Neumann et al., 1999 and Miller et al., 2001) and phytases on the other hand (Li et al., 1997 and Richardson et al., 2001).

Cluster root developmental stages in white lupin

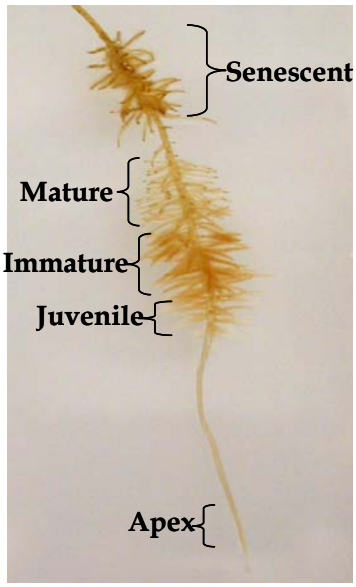

Figure 6: Cluster root stages in white lupin

Four stages can be differentiated in white lupin cluster roots grown in hydroponic conditions

(Picture: N. Langlade)

In white lupin, formation of cluster roots follows a well-defined developmental pattern (Figure 6) with different stages which last for only few days. Young, growing cluster roots release mainly malate and only low amounts of citrate. Immature cluster roots are fully grown and secrete little amounts of citrate and malate. The mature stage of cluster roots is characterized by a high exudation of citrate and a concomitant acidification of the root environment. Finally, at the senescent stage, the pH increases again and secretion of organic acids ceases (Massonneau et al., 2001). While in plants grown in hydroponic conditions, these stages can easily be differentiated, in soil-grown plants, the distinction between juvenile and immature cluster roots is not possible due to the similar pH value of the environment surrounding these two root stages and to the fact that the soil prevents the observation of the root morphology.

1.3. ROOT-INDUCED CHANGES IN THE SURROUNDING SOIL: THE RHIZOSPHERE CONCEPT

1.3.1. The rhizosphere: definitions

Due to its respiration, water and nutrient uptake, as well as its secretion of organic compounds, the plant root system alters greatly the physical and chemical conditions in the surrounding soil. Thus, the soil-root interface represents a very particular environment, which was named "rhizosphere" by Hiltner a little more than a hundred years ago. The definition which is now generally accepted is that rhizosphere corresponds to the volume of soil under the influence of the root, as well as to the root itself (Lynch, 1990). The rhizosphere can be further subdivided into two fractions (Gobat et al., 1998): i) the root tissues and surface, referred to as "rhizoplane-endorhizosphere" or "RE" and ii) the adhering soil, which is called "rhizosphere soil" or "RS". In the present study, these two levels of root proximity will be differentiated and compared with the non rhizosphere soil, the soil which is not in contact with the root and which will be called "bulk soil" or "BS".

1.3.2. The rhizosphere of white lupin cluster roots

Due to the high secretion activity of white lupin cluster roots, the root-induced changes occurring in the rhizosphere will be very important, as exemplified by the pH decrease around mature cluster roots (Figure 7), which is still visible through a one centimeter thick buffering rhizosphere soil layer. These chemical changes occur very rapidly, since the cluster root stages only last for a few days and they are likely to influence the microbial communities living in the rhizosphere.

Figure 7: Rhizosphere acidification by mature cluster roots

White lupin root system was overlaid by an agar gel containing a pH indicator. Yellow zones indicate acidification and correspond to the mature cluster roots.

1.3.3. Plant microbe interactions

The potential plant microbe interactions which could take place in the rhizosphere of white lupin cluster roots are discussed in details in the introduction of chapter 3. However, we will briefly mention here the most important processes which are likely to occur between white lupin cluster roots and the associated microflora. These potential interactions are described in Figure 8. Both the plant and the microflora rely on organic acids and phosphatases to acquire phosphate form the inorganic and the organic pool. One can hypothesize that plant- and microbe-solubilized Pi will then be available, once in the soil solution, to both the plant and the microbes, but it is not known whether one "direction" (microbial uptake of plant-released Pi *vs.* plant uptake of microbe-released Pi) is favored. On the one hand, heterotrophic rhizosphere bacteria and fungi might use the secreted citrate and malate as a carbon source, thus lowering the efficiency of the plant P acquisition.

On the other hand, secretion of phenolics by cluster roots (Neumann et al., 2000), as well as the drastic pH decrease around mature cluster roots should decrease the bacterial population levels and thus reduce this organic acid degradation effect. Auxin and siderophore production are well-known features of so-called "PGPRs" (plant growth promoting rhizobacteria) and in the case of white lupin, production of auxin seems of particular interest due to the role of this phytohormone in cluster root formation.

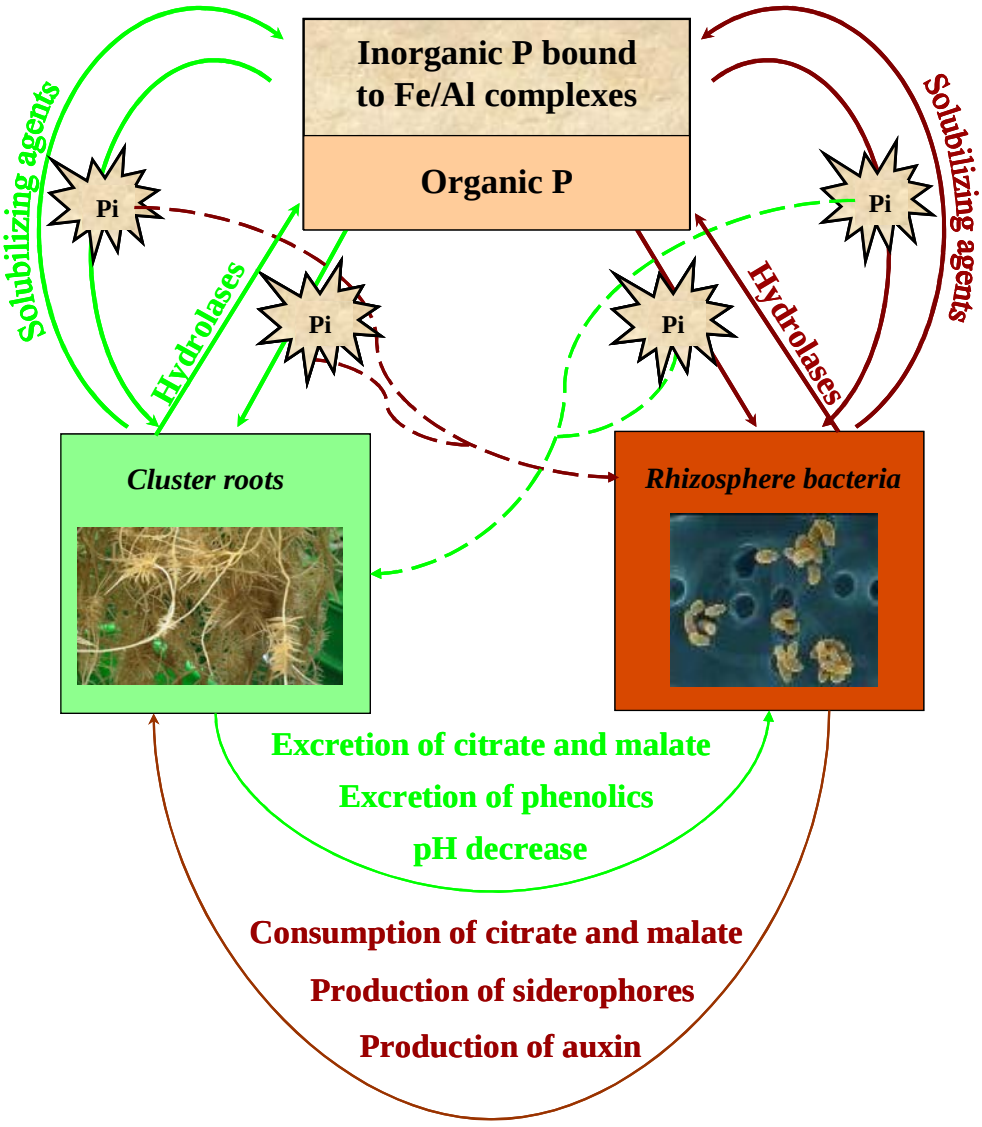

Figure 8: Potential plant microbe interactions in the rhizosphere of white lupin cluster roots

1.4. PRESENTATION OF THIS WORK: MAIN QUESTIONS AND OBJECTIVES

1.4.1. General aim and overview of the thesis

The present work aims at improving our understanding of white lupin P acquisition by studying cluster root secretion physiology and investigating the cluster root-induced changes in the associated rhizosphere microflora.

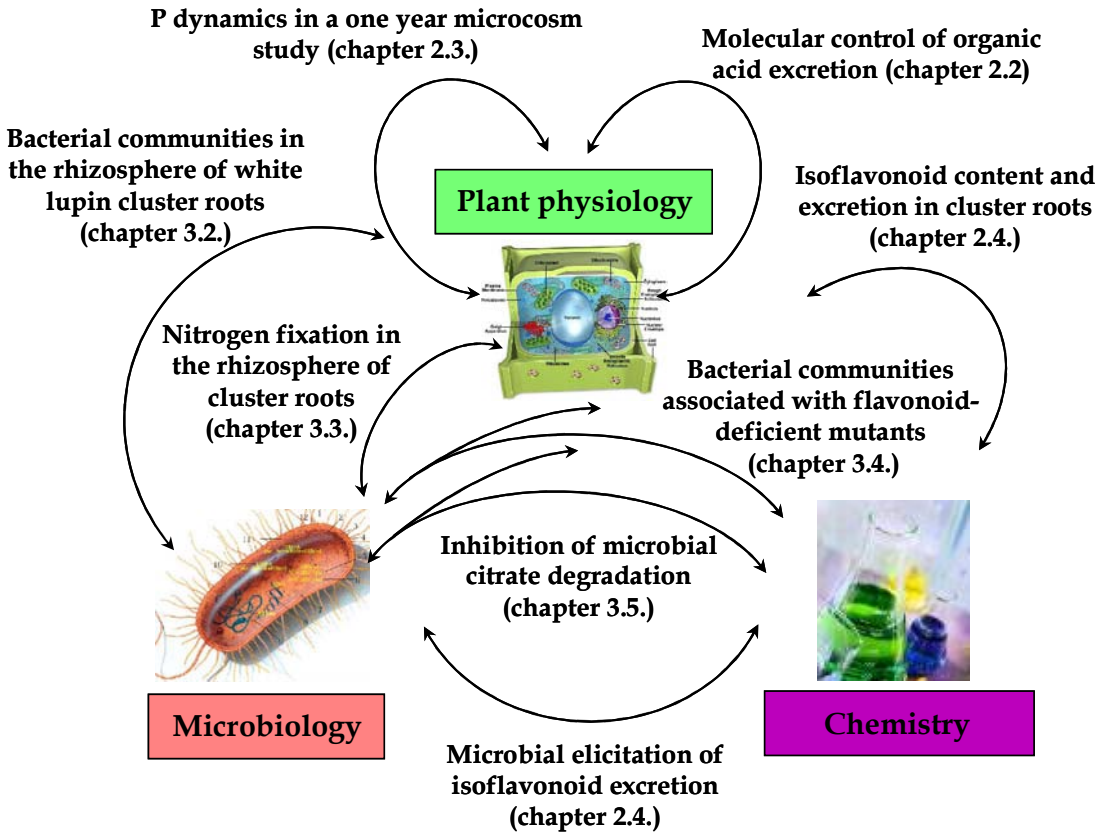

Figure 9: Network of experiments

Figure 9 illustrates the network of experiments conducted to achieve this general aim. Investigations have involved three main domains: plant physiology, microbiology and chemistry. While few experiments focused on a single domain (for instance chapter 2.2.: molecular control of organic acid secretion), most of them were based on the interaction of two or three domains, like the last section, 3.5., integrating chemically analysed

isoflavonoids, plant extracellular enzymatic antifungal activities, as well as root-induced changes which lower bacterial abundance in the rhizosphere of mature cluster roots.

The thesis is structured as follows: after the introduction, a first experimental part (chapter 2) is devoted to the study of root secretion physiology and P uptake. In this chapter, we first investigate the molecular control of organic acid secretion and especially the potential role of a specific enzyme, ATP citrate lyase, in organic acid metabolism (2.2.). The second part (2.3.) assesses the effect of these organic acids and of other P acquisition mechanisms on P dynamics in a microcosm study and finally, the last part (2.4.) deals with the attempt to extend the knowledge on root secretion physiology beyond organic acids by investigating the secretion of isoflavonoids in different stages of white lupin cluster roots.

The second experimental part (chapter 3) concerns the plant microbe interactions in the rhizosphere of *L. albus* and *A. thaliana* and attempts to fill up the gap of knowledge which very long affected cluster root physiology, namely the associated microflora. The first section (3.2.) describes the bacterial communities associated with white lupin cluster roots in terms of abundance, diversity, function and community structure. A short report on nitrogen fixation adds another important aspect to the picture of plant microbe interactions in the rhizosphere of cluster roots (3.3.). To investigate the potential impact of flavonoids on the structuring of bacterial rhizosphere communities, we used *A. thaliana* mutants deficient in flavonoid synthesis and compared the bacterial communities associated with the wild type and the mutant (3.4.). Finally, the last section (3.5.) integrates aspects of several domains to illustrate how white lupin inhibits microbial degradation of the secreted P-solubilizing agents.

Due to the diversity of the various topics investigated, each chapter is preceded by an introduction and ends up with a synthesis and a discussion. A global synthesis and outlook (chapter 4) closes up this work.

1.4.2. Collaboration and context of the study

This Ph.D. thesis was financed by the Swiss national foundation in the frame of the NCCR (national center of competence in research) "Plant Survival" and affiliated to the project 4 "Plant nutrition under stress conditions". Project 4 was directed by Prof. K. Föllmi (Institute of Geology, University of Neuchâtel). For the common experiments of the NCCR 4 project, collaborations were established with Dr. C. Le Bayon and Prof. J.-M. Gobat in soil biology (University of Neuchâtel), with Prof. U. Feller and Ph.D. student V. Page (University of Bern) for the transport of heavy metals in lupin and wheat and with Prof. K. Föllmi, Dr. V. Matera and Dr. T. Adatte (Institute of Geology, University of Neuchâtel) for the rock-soil processes. Results from this common experiment are presented in chapter 2.3. (P dynamics in a microcosm study). Moreover, data analysis for a long-term comparison of the bacterial communities associated with white lupin and wheat is still ongoing and will give raise to an independent publication in collaboration with the Microbiology department and the Soil Biology Department of the University of Neuchâtel.

For all other experiments, collaborations were established with the Microbiology Department (Prof. M. Aragno and Dr. N. Fromin) and the Analytical Chemistry Department (Prof. R. Tabacchi and Dr. E. Abou-Mansour). Further people involved were S. Plaza, with whom I performed the work on the molecular control of organic acid secretion (2.2.) and C. Theurillat, who did most of the practical work related to nitrogen fixation in the rhizosphere of white lupin (3.3.) during her 6 weeks practical stay in the Microbiology Department.

CHAPTER II

Cluster root secretion physiology and phosphate acquisition

Chapter II: overview

Chapter II: overview (continued)

2.1. INTRODUCTION

2.1.1. Chapter presentation

In the following chapter, we investigated the secretion physiology of white lupin cluster roots in order to better understand the mechanisms underlying the tolerance to soils where phosphate is sparingly available. We analyzed the involvement of a particular enzyme, ATP citrate lyase (ACL), in the secretion of organic acids in white lupin cluster roots (2.2.), conducted a study in microcosms to monitor P uptake and dynamics in soil-grown lupins during a one year cultivation period (2.3.) and characterized the isoflavonoids produced in and secreted from white lupin cluster roots (2.4.).

2.1.2. Molecular control of organic acid secretion

In the first part of this chapter (2.2.), we characterized the possible role of an enzyme, ATP citrate lyase (ACL), in the shift of organic acid from malate to citrate during cluster root development. ACL (EC 4.1.3.8) catalyses the formation of oxaloacetate and acetyl-CoA from citrate and Coenzyme A with a concomitant hydrolysis of ATP. Since oxaloacetate can readily be transformed into malate, this enzyme constitutes a link between citrate and malate, the two major organic acids secreted by white lupin cluster roots. This work had been initiated by N. Langlade during his Ph.D. thesis (2002). He measured ACL expression and activity and observed that they were highest at the juvenile stage of cluster roots. He could show a strong correlation between malate secretion by cluster roots and activity of ACL and this led to postulate a new role for ACL, namely the shift in organic acid preferentially secreted in white lupin cluster roots (Langlade et al., 2002). This work is reported in chapter 2.2.2. As a follow-up of N. Langlade's work, we wanted to see if the hypothesis of ACL's involvement in the shift of organic acid secretion in white lupin could be verified by overexpressing this enzyme in *Arabidopsis thaliana* and analyzing the production and secretion of citrate and malate in the transformed plants and in the corresponding wild-type (2.2.3.). The plants overexpressing ACL should, if Langlade's hypothesis is confirmed, secrete more malate

than the wild type. In addition, it was originally planned also to engineer plants over-producing and secreting citrate by reducing the expression of the aconitase gene through a silencing approach. Knocking out an enzyme of the TCA cycle appeared to be too drastic a method and this is why we attempted to silence the aconitase by transforming plants with a vector overexpressing 700 bp of the aconitase gene in the antisense orientation. The idea was that upon gene expression in the cell, the mRNAs of the transgene would hybridize with the mRNAs of the original gene and form a double-stranded RNA, which would be degraded by the cell. With these two kinds of plants (ACL overexpressing and malate secreting on the one hand and aconitase silenced and citrate secreting on the other hand), we planned to perform microbial community studies, to see which changes occurred in the structure of associated microbial communities when one particular organic acid was secreted in higher amounts. Unfortunately, this could not be done because the secretion of malate by ACL overexpressing plants was not enhanced as compared to the wild-type (see chapter 2.2.2) and because the transformation of *A. thaliana* with the antisense aconitase appeared to be unsuccessful or lethal for the seeds (no seed could be selected on plates containing the corresponding antibiotic resistance).

As for ATP citrate lyase, this enzyme presented another interest for us, in addition to the indirect production of malate, namely the supply of acetyl-CoA, which can be used for membrane biosynthesis, but also for the flavonoid biosynthetic pathway. Therefore, the characterization of ACL constitutes a link between the investigation of the molecular control of organic acid secretion (2.2.2.) and the chapter dealing with the characterization of the isoflavonoid secretion pattern in growing cluster roots (2.2.4.).

2.1.3. Studying P uptake in microcosms: between hydroponics and field conditions

Even if the molecular mechanisms leading to the enhanced secretion of organic acids at the mature stage of cluster roots are still not fully understood, it is nevertheless well established that these organic acids, in addition to the secretion of phosphatases and to

rhizosphere acidification in calcareous soils, constitute the key element of white lupin's P acquisition strategy. However, until now, most of the studies devoted to this subject were performed in hydroponic culture or, rarely, in sandy substrates, for a better understanding of the processes involved.

In the second part of this chapter (2.3.), we present a study performed with an experimental design that aimed at investigating P uptake by white lupin in conditions which would come nearer to the real field situation. White lupins were cultivated in large microcosms (containing 7 kg of soil) for one year (replanted every 8 weeks) and P dynamics was monitored at different time points and at different root proximity levels. For experimental reasons, it was not possible to separate the different cluster root stages and cluster roots were compared, as a whole, with non cluster roots with regard to P desorption, phosphatase activities and organic acid secretion.

2.1.4. Cluster root secretion physiology: more than "just" organic acids…

Last but not least, we undertook the characterization of the isoflavonoid profiles produced in and excreted from growing cluster roots (2.4.), in order to extend our knowledge of the secretion physiology beyond organic acids. We focused on this particular class of secondary compounds, which has been shown to be involved both in nutrient acquisition (nitrogen and iron) and in plant microbe interactions (attraction of nitrogen-fixing bacteria or antimicrobial activity against fungi).

With the help of chemists of the University of Neuchâtel (Dr. E. Abou-Mansour and Prof. R. Tabacchi), we were able to identify the major isoflavonoids present in and secreted from white lupin cluster roots and we investigated the effect of phosphorus supply, root type and cluster root stage on this production and secretion of isoflavonoids (2.4.2.). This is to our knowledge the first report on phenolics secreted by cluster roots in any plant species and constitutes an important progress towards a more complete understanding of cluster root secretion physiology.

In a second step, we addressed the question whether this isoflavonoid secretion would be influenced by rhizosphere micro-organisms and we tested the elicitation effect of several bacterial and fungal strains on isoflavonoid secretion by white lupin seedlings. Isoflavonoids have been assimilated to phytoalexins, compounds which very rapidly accumulate upon challenge with a pathogen and this led us to think that isoflavonoid secretion in white lupin might be influenced by rhizosphere bacteria and fungi. This was indeed the case, as reported in chapter 2.4.3., and even if the results should be confirmed because only one experiment without replicates was carried out, they give interesting preliminary indications that white lupin reacted strongly and strain specifically to bacterial and fungal elicitation.

2.2. THE POTENTIAL ROLE OF ATP-CITRATE LYASE IN ORGANIC ACID METABOLISM

2.2.1. Introduction

In response to P deficiency, white lupin cluster roots excrete high amounts of citrate. The metabolic changes in the root cells leading to citrate accumulation and secretion have been investigated in white lupin in several studies (Kihara et al., 2003; Neumann et al., 2000; Uhde-Stone et al., 2003). They are summarized in figure 10.

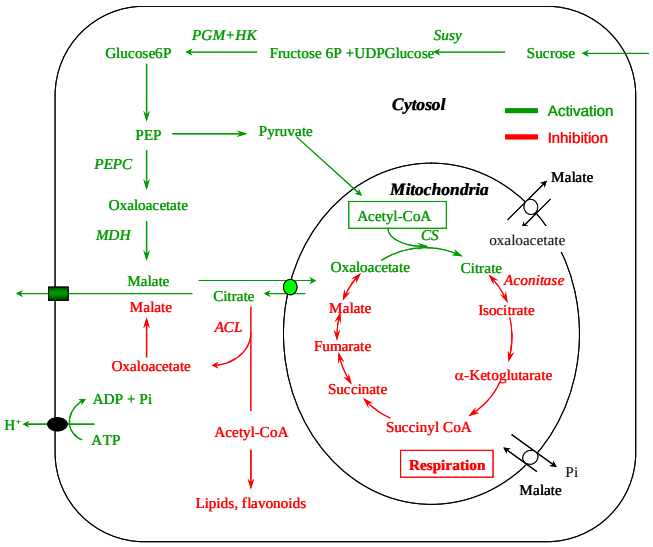

Figure 10
(modified from Neumann and Martinoia, 2002)

Model for P deficiency-induced metabolic changes related to accumulation and exudation of citrate in cluster roots of Lupinus albus. *Stimulation of metabolic reactions and sequences are marked in green, inhibition are marked in red. Abbreviations: ACL, ATP-citrate lyase; CS, citrate synthase; HK, hexokinase; MDH, malate dehydrogenase; PEP, phosphoenol pyruvate; PEPC, phosphoenolpyruvate carboxylase; PGM, phosphoglucomutase; Susy, sucrose synthase*

Among all the enzymatic steps involved in carboxylate metabolism in growing cluster roots, we decided to focus on one particular enzyme, ATP-citrate lyase (ACL), which was of particular interest to us for different reasons: i) we identified it as differentially expressed in growing cluster roots (Langlade et al., 2002), ii) this enzyme constitutes a link between citrate (substrate) and malate (reduced from the product oxaloacetate), the tow major carboxylates secreted by cluster roots and iii) the acetyl-CoA produced by ACL could be involved in isoflavonoid metabolism (see chapter 2.4.) in addition to the biosynthesis of membrane lipids. ATP citrate lyase transforms citrate and Coenzyme A into oxaloacetate and acetyl-Coenzyme A using ATP as energy source (Figure 11). The localization and function of this enzyme in plants is still not completely elucidated. The

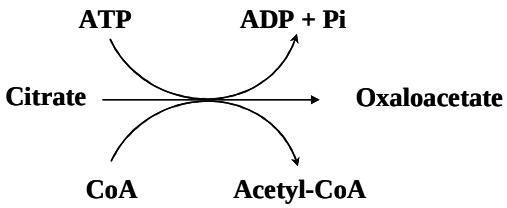

Figure 11: reaction of ATP-citrate lyase

reaction products of the ACL activity, oxaloacetate and acetyl-CoA, can both be used in different biosynthetic pathways. The main metabolic pathway studied so far is the relationship between ACL activity and lipid synthesis through production of acetyl-CoA (Fatland et al., 2000 and Rangasamy and Ratledge, 2000). In contrast, the involvement of ACL generated acetyl-CoA in the biosynthesis of flavonoids has not been studied as intensively, even if Fatland et al. (2002) postulated that, due to a cytosolic localization of ACL, the acetyl CoA produced by this enzyme would be used for fatty acids elongation and flavonoid biosynthesis. In addition to these roles, Langlade et al. (2002) presented a putative novel function of ACL consisting in a switch from malate to citrate during cluster root development in white lupin. At a particular developmental stage, ACL expression is downregulated and a switch from malate to citrate occurs in the secretion pattern. In primary roots of white lupin and maize, ACL activity was positively correlated with malate exudation, meaning that in addition to lipid biosynthesis, in plants, ACL may be implicated in malate production and secretion. As for the structure, in contrast to the situation in humans, the plant ACL is constituted of two polypeptides, ACLa and ACLb, encoding the N- and C-terminus of the enzyme (Langlade et al., 2002, nomenclature) and is encoded by two different genes (Kanao et al., 2001; Langlade et al., 2002).

In this chapter, we present the molecular cloning, characterization, and heterologous expression of the lupin ATP citrate lyase (Langlade et al., 2002: part 2.2.2.). We also provide first indications that this enzyme might be involved in the shift observed between malate and citrate when the juvenile cluster roots reach the mature stage. In the next part (2.2.3.), we report the overexpression of the lupin ACL in tobacco and *Arabidopsis* plants and the secretion of organic acids and phenolic compounds by these transformed plants.

2.2.2. ATP-citrate lyase: cloning, heterologous expression cloning, heterologous expression and possible implication in root organic acid metabolism and secretion.

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By ***Nicolas Bernard Langlade****, Gaëlle Messerli, Laure Weisskopf, Sonia Plaza, Jana Smutny, Günter Neumann, Enrico Martinoia and Agnès Massonneau*

2.2.2.1. Abstract

In order to cope with phosphate deficiency, white lupin produces bottle-brushed like roots, so-called cluster or proteoid roots which are specialized in malate and citrate secretion. Young, developing cluster roots mainly secrete malate whereas mature cluster roots mainly release citrate. Mature proteoid roots secrete four to six times more carboxylates compared with juvenile proteoid roots. Using a cDNA-amplified restriction fragment length polymorphism (AFLP) approach we identified a gene coding for a putative ATP-citrate lyase (ACL) up-regulated in young cluster roots. Cloning of the lupin ACL revealed that plant ACL is constituted by two polypeptides (ACLA and ACLB) encoded by two different genes. This contrasts with the animal ACL, constituted of one polypeptide which covers ACLA and ACLB. The ACL function of the two lupin gene products has been demonstrated by heterologous expression in yeast. Both subunits are required for ACL activity. In lupin cluster roots, our results suggest that ACL activity could be responsible for the switch between malate and citrate secretion in the different developmental stages of cluster roots. In primary roots of lupin and maize, ACL activity was positively correlated with malate exudation. These results show that ACL is implicated in root exudation of organic acids and hence plays a novel role in addition to lipid synthesis. Our results suggest that in addition to lipid biosynthesis, in plants, ACL is implicated in malate secretion.

Please refer to the appendix for the full version of this publication

2.2.3. Overexpression of the lupin ACL gene in *Arabidopsis thaliana* and *Nicotiana tabacum*

2.2.3.1. Abstract

In previous work (Langlade et al., 2002), we have shown that ATP citrate lyase activity was correlated with malate production and secretion in developing white lupin cluster roots. In order to obtain evidence for this putative new role of ACL in organic acid metabolism, we overexpressed the lupin ACL gene in *Arabidopsis* and tobacco plants and measured the secretion of organic acids in the transformed plants. We also designed a construct with a GFP fused to the N terminus of the lupin ACL, in order to elucidate the subcellular localization of this enzyme. Our results showed that the enzyme was situated at the plasma membrane, a localization which until now has never been found for this enzyme. With respect to the organic acid secretion, the results obtained support the hypothesis of a potential role of ACL in malate secretion, but additional data are needed for a conclusive proof of this role. In addition to ACL's potential role in malate production, an increase in phenolic compounds secreted by ACL overexpressing *Arabidopsis* plants also suggested that ACL might be involved in the production and secretion of phenolic compounds. The fact that young cluster roots, where ACL activity is highest, are the stage where high amounts of isoflavonoid are produced and secreted is consistent with this hypothesis.

2.2.3.2. Introduction

ATP citrate lyase catalyses the ATP-dependent transformation of citrate into oxaloacetate, with a concomitant production of acetyl CoA from Coenzyme A (see Figure 11). Langlade and collaborators (2002) found that the white lupin ACL was constituted of two subunits both required for enzyme activity and corresponding to the N- and C-terminals of the mammalian ACL. This structure involving two subunits had also been found in *Arabidopsis thaliana* (Fatland et al., 2002). Until now, the role of ACL has been often related to the production of acetyl-CoA needed for lipid biosynthesis (Fatland et al., 2000; Rangasamy and Ratledge, 2000). In the preceding chapter (2.2.2.), it has been shown that in white lupin, ATP citrate lyase (ACL) activity was correlated with malate production and secretion. This observation led to postulate a new role for ACL, namely the regulation of the balance between citrate and malate. Since oxaloacetate is readily reduced to malate, ACL represents a link between citrate and malate, the two major organic acids secreted by white lupin's cluster roots. The upregulation of ACL in young cluster roots and the following downregulation at the mature stage of cluster roots nicely correlates with the high malate secretion and high citrate secretion which characterize juvenile and mature cluster root stages, respectively. In addition, the overexpression of ACL at the juvenile stage correlates also very well with the enhanced secretion of isoflavonoids (see chapter 2.4.2.), which also depend on the presence of acetyl CoA for their synthesis (Figure 10). However, to obtain a final evidence of ACL's role in malate or isoflavonoid production and secretion and to be able to positively confirm this observed correlation, additional experiments are needed. The present chapter describes the experiments undertaken to attempt to provide additional evidence for this new role of ACL. The most straightforward approach in such questions usually is to overexpress the enzyme of interest and to look for the corresponding phenotype in transformed plants. Since this cannot be done in white lupin, we used *Arabidopsis thaliana*, as well as *Nicotiana tabacum* to perform this overexpression study of the lupin ACL. Transformation protocols were available for both plants. *A. thaliana* was chosen as the genetic model of choice, while tobacco was used for its high biomass production and nice growth in hydroponic culture systems. The two subunits of the lupin ACL were

introduced in a stable transformation into both *A. thaliana* and tobacco plants. The expression levels of the gene were assessed in positive transformants and analysis of organic acid and phenolic secretion were performed. To increase levels of secreted organic acids, two treatments were used: i) the addition of aluminium to the medium, which has been reported to induce synthesis of organic acids, mainly citrate and oxaloacetate (Gaume et al., 2001b; Ligaba et al., 2004; Ma et al., 2001) and ii) the growth in P deficient conditions, which is known to induce increased secretion of carboxylic acids (Neumann and Römheld, 1999). In addition, subcellular localization of the enzyme, which provides information about the possible functions of the enzyme, were also performed in *A. thaliana* transformed with a stable construct containing a GFP fused to the N-terminal subunit of the ACL gene.

2.2.3.3. Material and methods

Insertion of ATP Citrate Lyase (ACL) into a vector suitable for stable transformation of Arabidopsis thaliana

Antisense orientation

ACLa was removed from the pGEMT-easy plasmid and introduced into pCAMBIA 1301 via digestions using *Bgl*II and *BstE*II. By this double digestion, we removed the GUS and replaced it by *ACLa*. For *ACLb*, different subcloning steps were necessary. The first step was the cloning of *ACLb* into the L42 plasmid. This vector was digested by *Nco*I, made blunt-end by Klenow fill-in and subsequently digested by *Pst*I. The temporary plasmid, 35Smod (see Figure 12 for the engineering of this vector) was first digested by *Sma*I (blunt ends) and subsequently digested by *Pst*I.

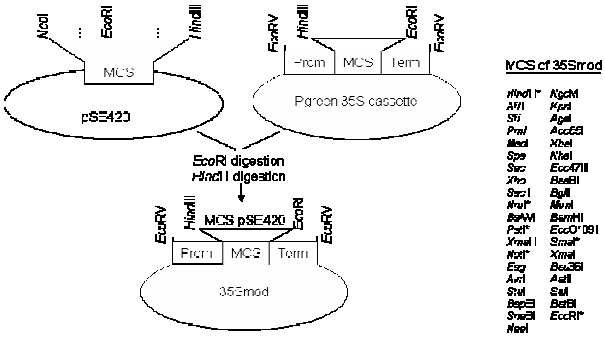
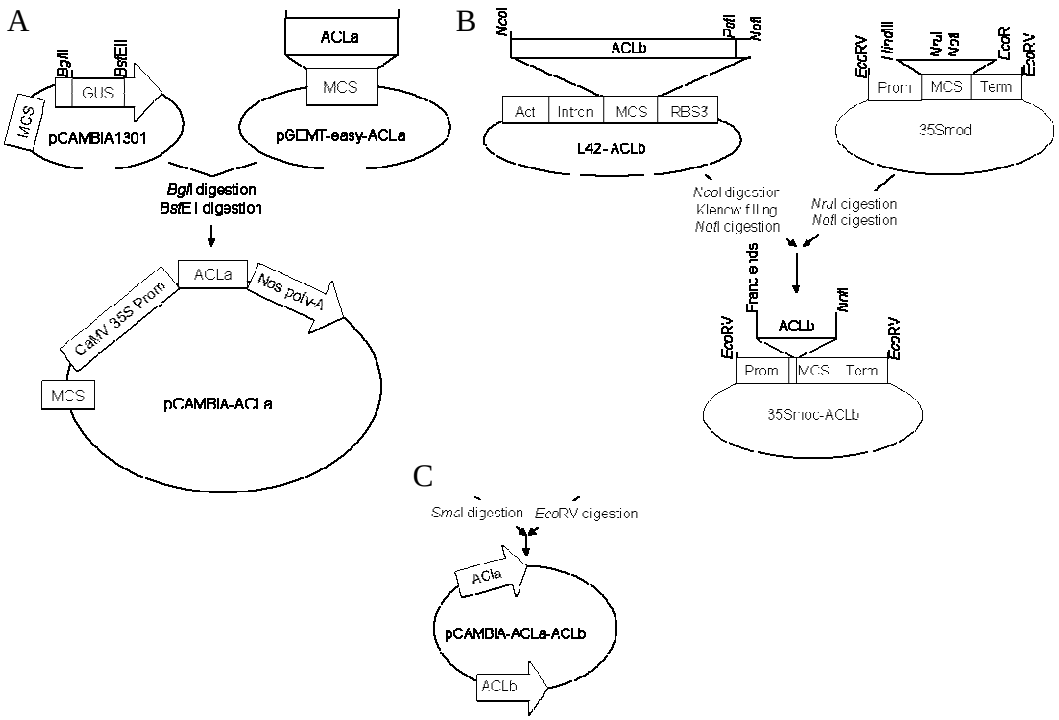


Figure 12: Construction of 35Smod vector

The MCS (multicloning site) of pSE420 was partially removed (from EcoRI to HindIII) and introduced into pGreen 35S. The entire list of unique restriction sites introduced from pSE420 is shown. The asterisk indicates that this enzyme has been tested and is a unique restriction site.

Figure 13: Construction of pCAMBIA-ACLa-(ACLb)as

A. The GUS was substituted by ACLa in the 35S-CaMV promoter-terminator cassette (represented by an arrow) in pCAMBIA 1301, giving pCAMBIA-ACLa. B. ACLb is introduced in an antisense orientation in the 35S-CaMV cassette of the 35Smod vector, giving 35Smod-(ACLb)as. C. The final step was the introduction of the 35S-CaMV cassette containing AClb antisense in the pCAMBIA-ACLa, producing pCAMBIA-ACLa-(ACLb)as.



The construction obtained was 35Smod-(*ACLb*)as (Figure 13 B). The final step was the removal of the 35S-CaMV promoter-terminator cassette containing *ACLb* of the 35Smod-(*ACLb*)as construct. This step was performed using two different restriction enzymes for the insert (35S with *ACLb*) and the vector (pCAMBIA-*ACLa*) both resulting in blunt end fragments, *Eco*RV and *Sma*I, respectively. The resulting construct, pCAMBIA-ACLa-ACLb is constituted of the two subunits of *ACL*, each comprised in a 35S-CaMV promoter-terminator cassette, as shown in Figure 13 C.

Sense orientation

For the replacement of *GUS* by the subunit *ACLa*, we followed the same protocol than for the antisense. *ACLb* was removed from L42-ACLb by *Nco*I digestion, filled in by Klenow and digested by *Not*I to obtain a blunt end and a *Not*I cohesive end, respectively.

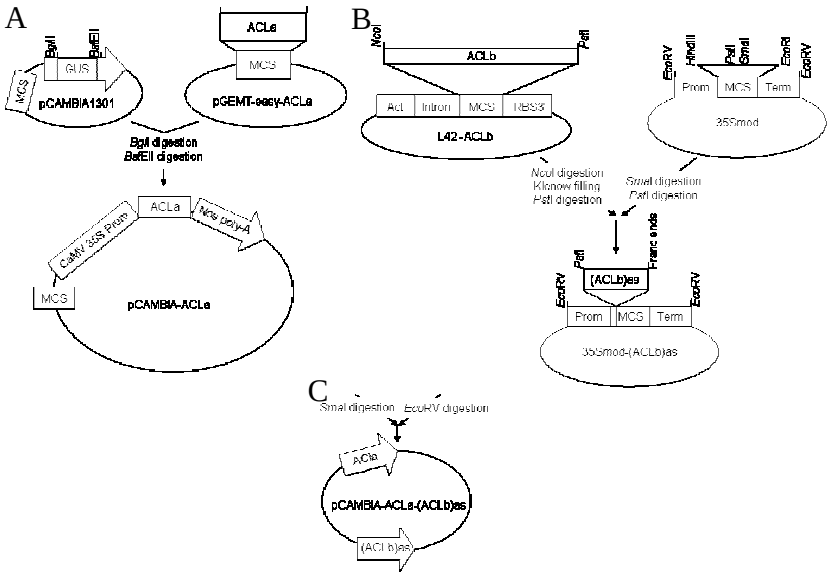


Figure 14: Construction of pCAMBIA-ACla-ACLb

A. The GUS was substituted by ACLa in the 35S-CaMV cassette (represented by an arrow) in pCAMBIA1301 giving pCAMBIA-ACLa. B. ACLb was introduced in sense orientation in the 35S-CaMV promoter-terminator cassette of the 35Smod vector, resulting in 35Smod-ACLb. C. The final step was the introduction of the 35S-CaMV cassette containing ACLb in the pCAMBIA-ACLa, producing pCAMBIA-ACLa-ACLb (C).

The intermediate plasmid, 35Smod, was digested by *Nru*I and *Not*I in order to obtain a blunt end and a *Not*I cohesive end, respectively. Once inserted into the 35S-CaMV promoter-terminator cassette, the entire cassette was removed by *Eco*RV, giving blunt ends, and introduced, as for the antisense, in pCAMBIA-*ACLa* digested by *Sma*I in the multicloning site of pCAMBIA1301 (Figure 14).

ACLb-GFP fusion, transfer into a 35S-CaMV cassette and final introduction into a vector containing ACLa

This cloning required different subcloning steps. The first step was the fusion of an enhanced *GFP* (EGFP) to *ACLb*. After this, *ACLb-GFP* was cloned into a 35S-CaMV cassette before to be cloned into the pCAMBIA 1301 containing the *ACLa* subunit (see above). For the amplification of EGFP (GFP6; Humair et al., 2001) primers containing an *Apa*I restriction site were designed. Sequences of the primers were 5'-CGA TGG ACC AGG TCA TGA GTA AAG GAG AAG AA-3' (forward primer) and 5'-CGA TGG ACC TCG TCT TTG TAT AGT TCA TCC AT-3' (reverse primer). Both the vector containing the *ACLb* (in pGEM T-easy) and the PCR product of the GFP were digested with *Apa*I and ligated to result in the vector called pGEMT-easy-*ACLb-GFP* (Figure 15 A). The absence of PCR errors was verified by sequencing. A PCR was performed on the *ACLb-GFP* fusion with primers containing the following restrictions sites: *Hind*III for the forward primer and *Kpn*I for the reverse primer. Sequences of primers were 5'-AGC AAG CTT ATG GCC AGA AAG AAG ATC AGA GAG-3' (forward primer) and 5'-GTT GGT ACC TTA AGC AGA AGC AGT GAT GCA CTG-3' (reverse primer). The amplified PCR product was digested by *Hind*III and *Kpn*I. A vector containing the 35S-CaMV promoter-terminator cassette (35Smod) was then digested with *Hind* III and *Kpn*I and ligated to form 35Smod-*ACLb-GFP* (Figure 15 B). The absence of PCR errors was verified by sequencing. The 35S-CaMV cassette containing *ACLb-GFP* was then excised with *Eco*RV and *Pvu*I, while the vector pCAMBIA1301 containing already the C-terminal subunit of the *ACL* (*ACLa*) under the control of a 35S-CaMV promoter) was also digested with *Eco*RV and subsequently with *Pvu*I and ligated (Figure15 C).

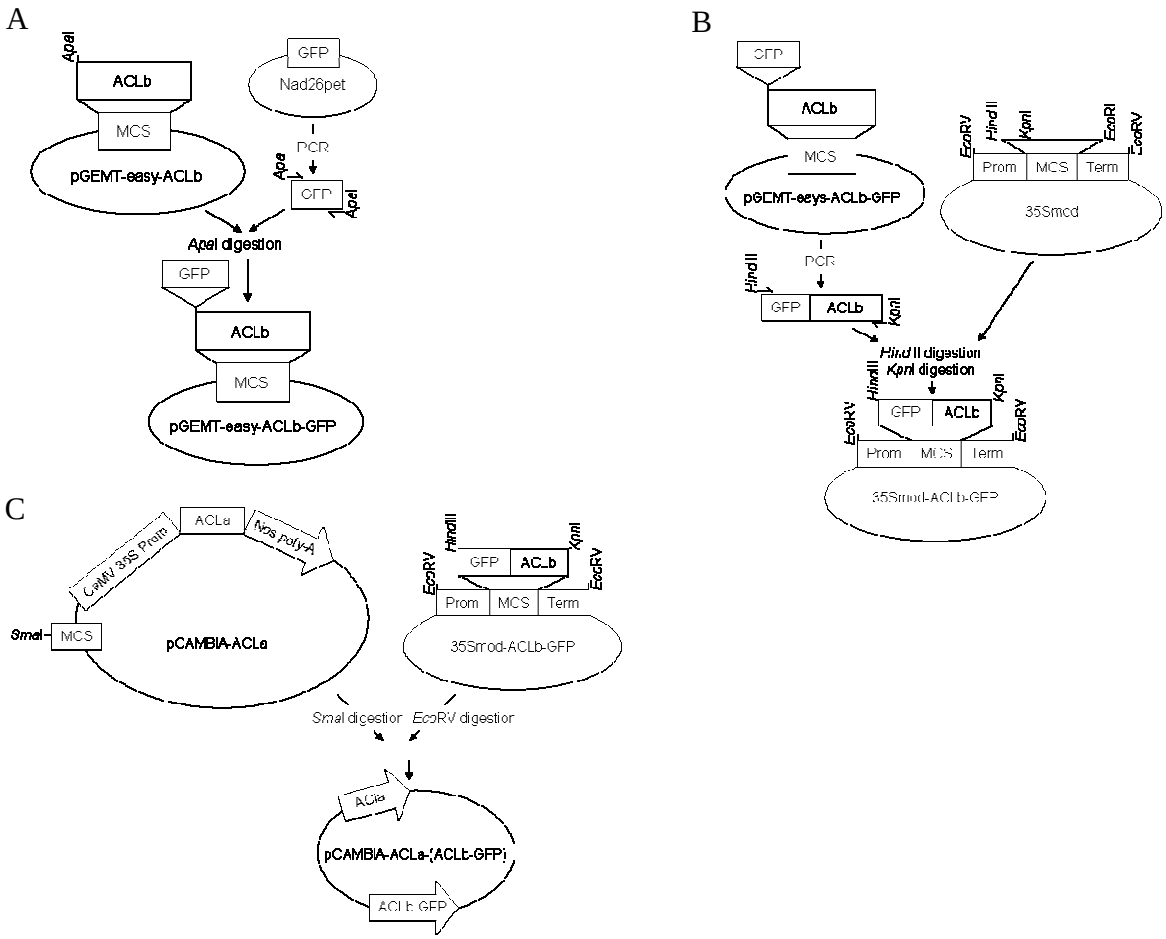


Figure 15: Construction of pCAMBIA-ACLa-(ACLb-GFP)

A. The first cloning step of the GFP ACLb was a digestion by ApaI restriction site in pGEMT-easy-ACLb, giving pGEMT-easy-ACLb-GFP. B. The ACLb-GFP was cloned into the intermediate vector, 35Smod. C. The final step was the cloning of the 35S-CaMV cassette containing ACLb-GFP into the pCAMBIA-ACLa resulting in pCAMBIA-ACLa-(ACLb-GFP).

Transformation of plants with ACL constructs

Transformation of *Nicotiana tabacum* was performed by EU partners (Prof. Danuta Maria Antosiewicz, University of Warsaw, Poland). Several homozygous transformant lines were selected on a medium with the specific antibiotic (ampicillin) and verified by RT-PCR analysis. Transformations of *Arabidopsis thaliana* with the different constructs

containing *ACL, ACLa*-(*ACLb* antisense), *ACLa-ACLb* and *ACLa*-(*ACLb-GFP*) were performed by floral dipping and transformants were selected on a medium with the specific antibiotic for pCAMBIA1301 (hygromycin), as described in Clough and Bent (1998). The expression of the ACL subunits in *Arabidopsis thaliana* was verified by RT-PCRs using the following primers (forward: 5'-TGG TCG ATG GAA AGC CTT TA-3' and reverse: 5'-CGG GTC CAT AAA CCT CAA TG-3')

Localization of pCAMBIA-ACLa-(ACLb-GFP) construct in young transgenic Arabidopsis *plants*

F_1 seeds were selected on plates containing hygromycin and positive, one week-old transformants were visualized by microscopy using a GFP specific filter. Plants with high expression levels of GFP were observed by Confocal Laser Scanning Microscopy (CLSM). GFP fluorescence was recorded using the corresponding filter sets and the images were colored using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

Physiological characteristics

Tobacco and *Arabidopsis* expressing the white lupin ACL were grown in hydroponic culture for six and nine weeks, respectively (for nutrient solution, see Massonneau et al., 2001). Different treatments like the addition of aluminium chloride (50µM $AlCl_3$ for 4 hours) or the absence of phosphate in the medium were tested to observe their effects on ACL activity and organic acid secretion. The plants were separated into shoot and root and weighted. The shoots were stored at -80°C for further analysis of the organic acid contents. The roots were incubated for one hour in water under gentle shaking to allow the release of root exudates. After one hour, the water containing the root exudates was frozen at -80°C, freeze-dried and resuspended in water prior to HPLC analysis. The roots were ground, centrifuged 10 minutes at 13000 rpm and 4°C and the supernatant was used to analyze the ACL activity (as performed by Langlade et al., 2002) and for organic acid

determination by HPLC analysis. The pH of the solution was measured. 4 replicates per line were used.

HPLC analysis of organic acids and phenolic compounds

Organic acids present in root tissues, as well as organic acids secreted during the hour of incubation in water were determined by HPLC analysis. For internal contents of organic acids, 50 µl of the supernatant obtained after centrifugation of the ground roots (see above) were injected into the HPLC column. For the secreted organic acid analysis, samples were resuspended after lyophilization in distilled water and 50 µl were injected. An anion-exchange column (BENSON polymeric^{TM} made of spherical sulfonated styrene-divinylbenzen resin in the H^+ form, with a porosity of 10 µm, a length of 300 mm and an internal diameter of 7.8 mm) was used to separate the different organic acids with an isocratic flow of H_2SO_4 20 mM at 0.7 ml.min^{-1}. Separation was achieved in 20 minutes and absorbance was monitored at 210 nm. Calibration curves with standard organic acids were performed for quantification of the most abundant organic acids present in the analyzed samples.

2.2.3.4. Results

Selection of transformants

Plants overexpressing *LaACLa-ACLb* were selected on selective medium and F_2 plants were used for the following experiments. Unfortunately, only very few plants expressing *LaACLa*-(*ACLb* antisense) were obtained and we did not have enough replicates to include this treatment in the analyses. Two different lines were used in the following experiments for each construction (tobacco expressing ACL, tobacco expressing empty vector and *Arabidopsis* expressing ACL) and were pooled in one treatment per construct after verification that there was no significant difference between them (Mann-Whitney U test of variance).

Presence of the LaACL in Arabidopsis thaliana

The presence of *LaACL* in transgenic *Arabidopsis thaliana* was verified by semi-quantitative RT-PCR. As house-keeping gene, S16 (At5g18380), was used as internal control. S16 encodes a 40S ribosomal subunit protein of *Arabidopsis thaliana* belonging to the S9P family of ribosomal proteins. Using specific primers for the ACLb subunit of white lupin, we detected a band of the expected size in the transgenic but not in the wild type plants (Figure 16). For *Nicotiana tabacum*, the presence was also controlled by RT-PCR by the polish group (data not shown).

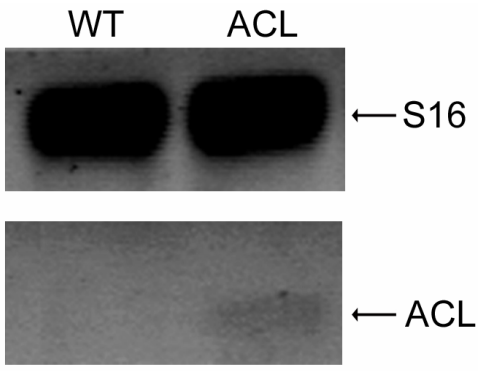


Figure 16:
RT-PCR on transgenic* Arabidopsis *expressing LaACLs

The presence of LaACLb was controlled by RT-PCR in Arabidopsis thaliana *wild type (WT) and transgenic plants (ACL). S16 as internal control and specific primers for ACLb were used.*

Localization of pCAMBIA-ACLa-(ACLb-GFP) in young transgenic Arabidopsis *seedlings*

Heterozygous seedlings transformed with *pCAMBIA-ACLa-(ACLb-GFP)* (F_1) were selected on hygromycin plates and subsequently analyzed by confocal microscopy. They showed high levels of fluorescence in both the roots and the leaves. In the roots, fluorescence was observed in the root epidermis (Figure 17 i and iv). Surprisingly, the fluorescence seemed to overlap with the plasma membrane as observed in the overlay figures (Figure 17 iii and vi). Cytoplasmic localization seemed unlikely due to the fact that vacuoles in young roots are small and therefore, our peripheral fluorescence could not correspond to the cytoplasm or the vacuolar membrane. In the leaves, fluorescence was observed in the inner membrane of the guard cells (data not shown) but we cannot exclude that the signal did not correspond to callose, which is known to accumulate in the cell wall of guard cells (Majewska-Sawka et al., 2002) and which could also be excited

by the excitation wavelength of the GFP (M. Klein, personal communication). A negative control with a callose specific staining would have been required to be able to better interpretate the localization results.

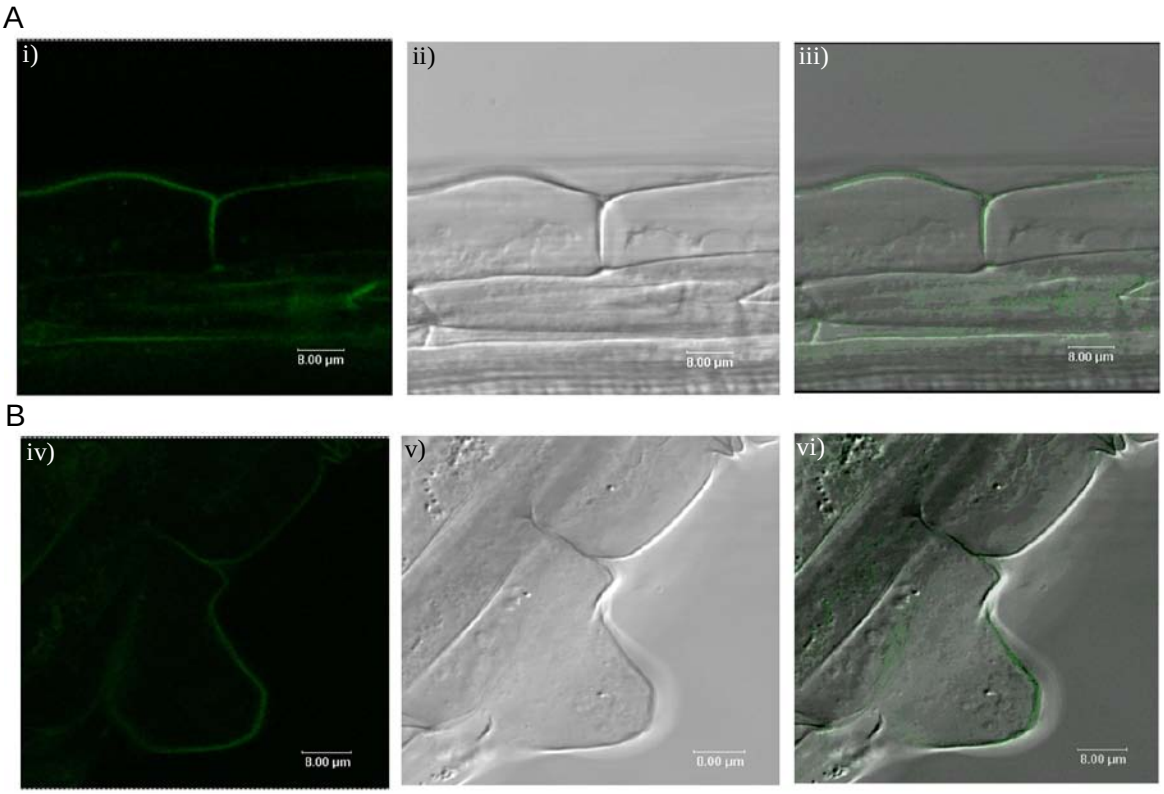


Figure 17:
Localization of pCAMBIA-ACLa-(ACLb-GFP) in young transgenic* Arabidopsis *plants

GFP-fluorescence emission (i and iv), bright field (ii and v) and overlay images (iii and vi) of a root epidermal cell (A) and a growing root hair (B). pm, plasma membrane.

Physiological characteristics of Nicotiana tabacum *overexpressing ACL*

Although the empty vector control showed very strange results for all the parameters studied and even if no significant differences could be shown between overexpressing and control plants, some trends could be observed: i) plant overexpressing ACL were bigger than the wild type (Figure 18 Ai), but no difference was observed when we plotted

the fresh weight shoot to root ratio (Figure 18 Aii); ii) ACL activity was higher in overexpressing plants than in the wild-type control and iii) the aluminium treatment increased the activity in both wild-type and overexpressing plants (Figure 18 B).

No difference was observed for the pH of the nutrient solution, which may either indicate that no exudation of organic acids and protons occurred or that the nutrient solution had a high buffer capacity.

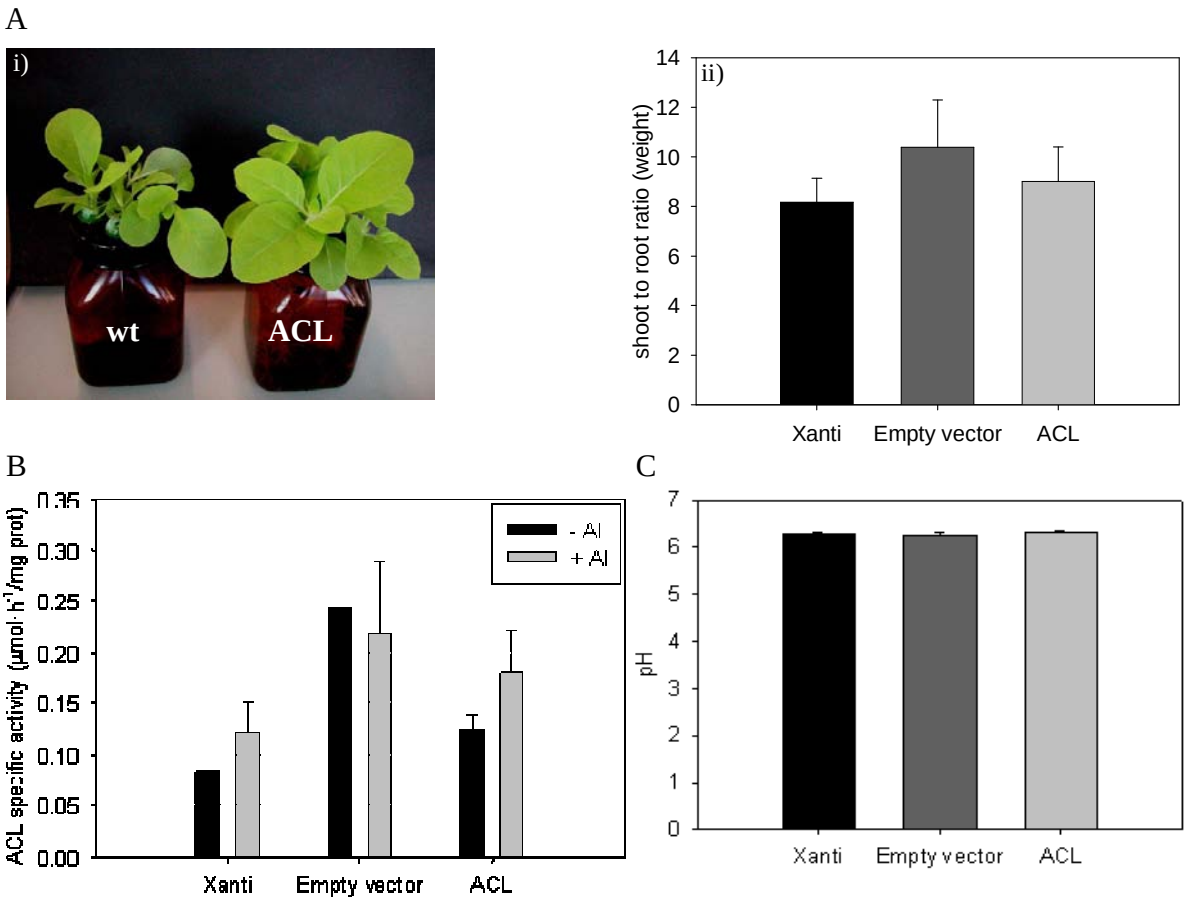


Figure 18: Physiological characteristics of tobacco plants overexpressing LaACL

A. Picture of hydroponically grown tobacco plants (i) and fresh weight shoot-root ratio of the different tobacco plants (ii). Xanti: wild type (Xanti), Empty vector: plants transformed with the empty vector) and ACL: ACL over expressing plants. B. ACL activity with (grey) or without (black) the addition of aluminium chloride for four hours before harvesting. C. pH of hydroponic solution one-week old in normal medium without aluminium chloride. The bars represent the means for the different treatments and the error bars represent the standard deviation.

HPLC analysis of organic acids and phenolic compounds in tobacco

To better characterize the synthesis and secretion of organic acids by roots of ACL overexpressing plant, HPLC analysis of organic acids was performed for both the internal contents and the secretion. By comparing the different spectra obtained for the external and internal compounds of tobacco, we observed the presence of four main compounds with retention times (RT) of 7.2, 11.5, 15.5 and 20 minutes corresponding to citrate (RT= 7.2), fumarate (RT = 15.5) and two phenolic compounds (RT = 11.5 and RT = 20 min) (see Figure 19 Ai for external and 19 Aii for internal secretion). The large peak eluting at 9.5 min unfortunately did not represent a single compound but was a mixture of one or two different molecules. Surprisingly, no malate was recovered from any tobacco sample.

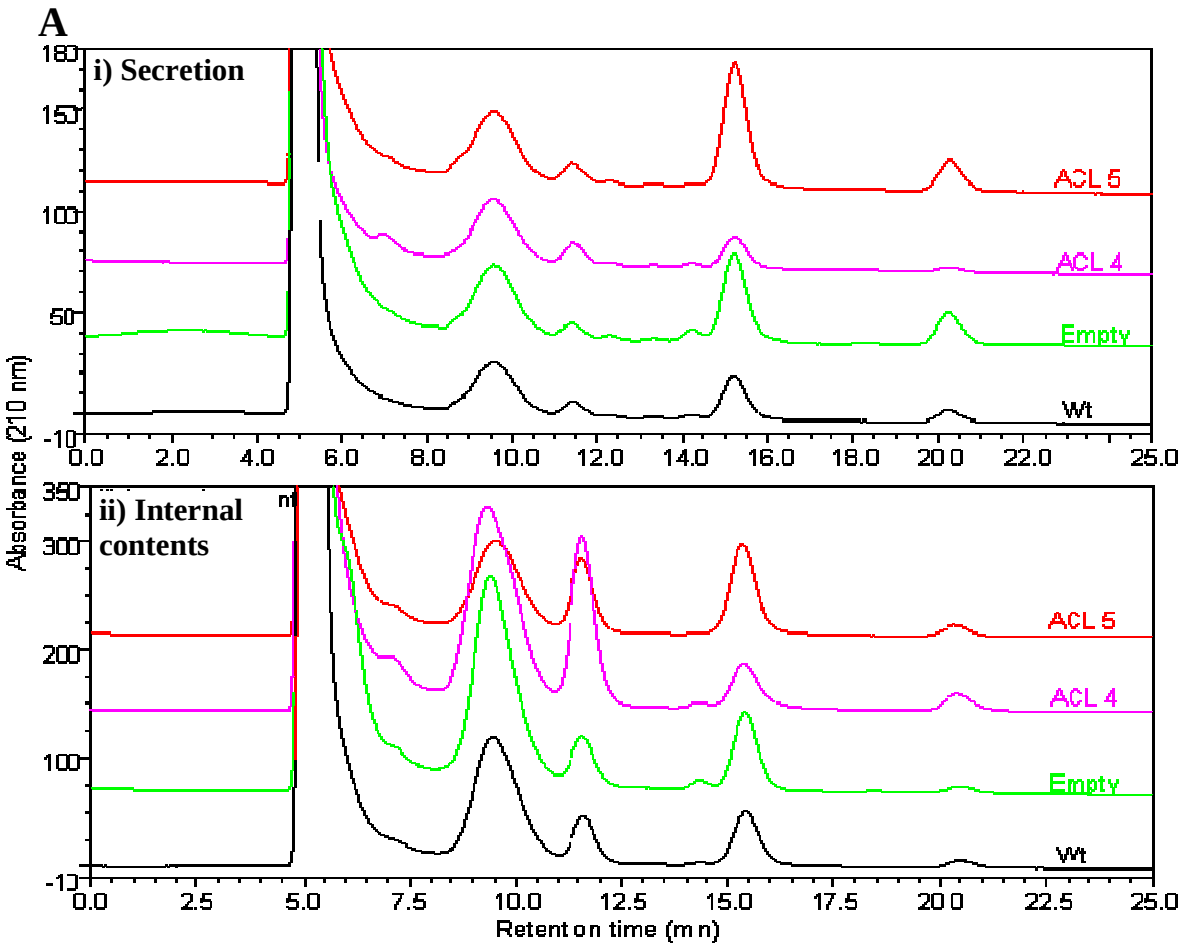


Figure 19: Organic acid secretion and content in tobacco expressing ACLa and ACLb

A. HPLC profiles for the secretion (i) and internal (ii) contents without aluminium treatment.

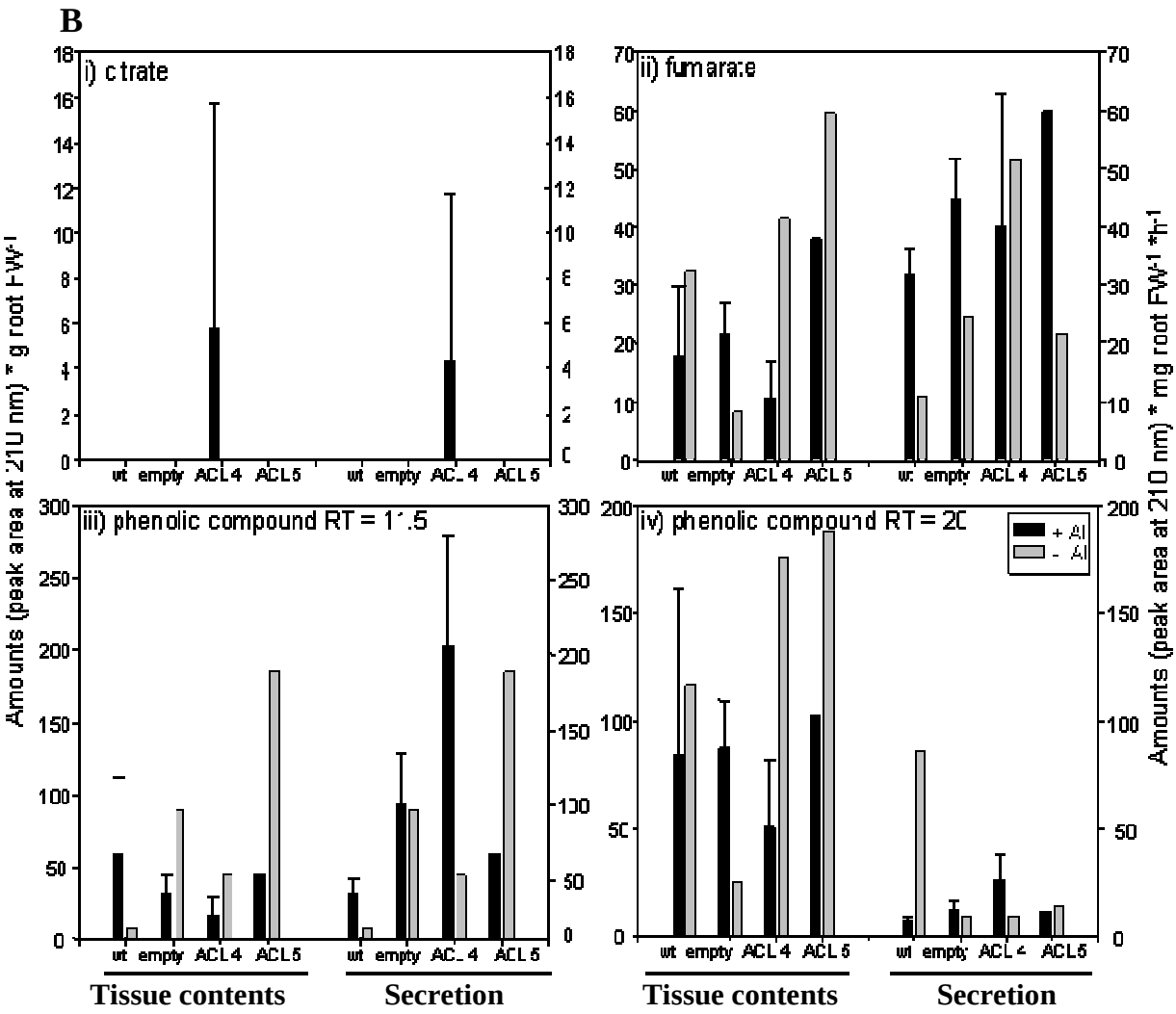


Figure 19 (cont.): Organic acid secretion and content in tobacco expressing ACLa and ACLb

B. Histogram representing the aluminium effect for the different main compounds represented by the peak area by the amount per gram of root fresh weight (left panel) and by the amount per gram of root FW and per hour (right panel) for citrate (i), fumarate (ii), phenolic compounds with an RT = 11.5 (iii) and 20 minutes (iv).

The quantification of the four major compounds described above showed following tendencies (Figure 19 B): i) citrate was only detected in presence of aluminium and in the overexpressing ACL line 4, where it was found in both internal contents and exudates (Figure 19 Bi); ii) fumarate was present in all the lines and could be detected both in the root tissues and in the exudates. Aluminium treatment induced a tendency to increase

secretion of fumarate. No significant difference between the lines was found, but fumarate levels were generally higher in ACL overexpressing plants as compared to the wild-type plants (Figure 19 Bii) and finally iii) aluminium treatment generally decreased the internal amounts of two phenolic compounds (RT = 11.5, Figure 19 Biii and RT = 20, Figure 19 Biv). The line 4 of ACL overexpressing plants showed an opposite effect in comparison with the other genotypes, with respect to the aluminium effect: whereas Al decreased the secretion levels of the two phenolic compounds in wild type plants and in the ACL line 5, it increased it in line 4.

Physiological characteristics of Arabidopsis thaliana *plants expressing enzymes involved in organic acid secretion*

No significant differences between wild type and ACL overexpressing plants could be shown for the shoot to root ration or the pH of the nutrient solution (Figure 20 A and B).

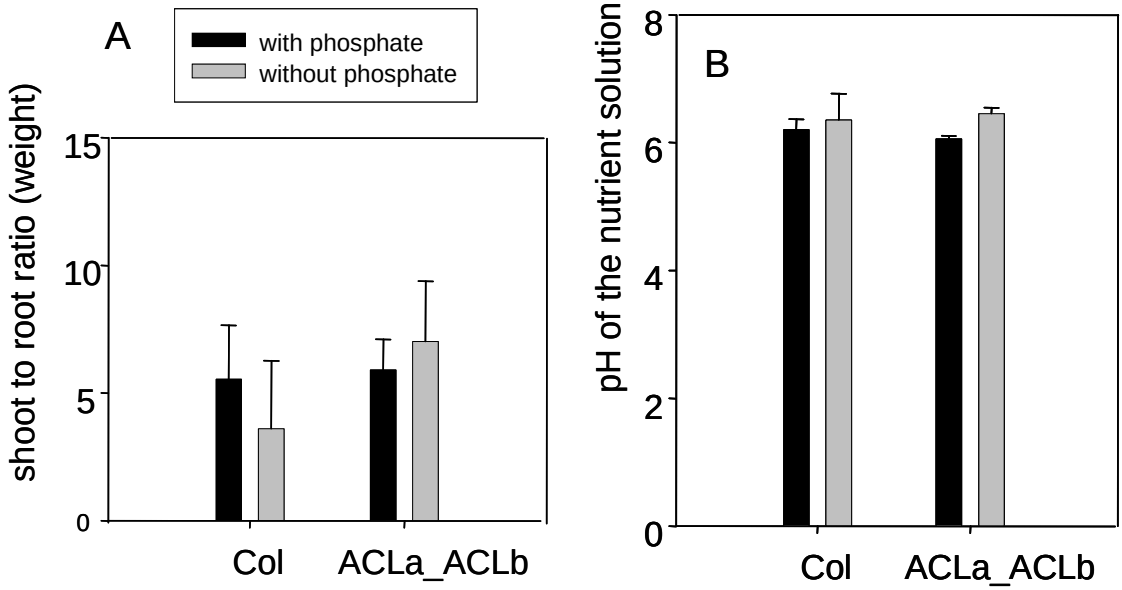


Figure 20: Physiological characteristics of* Arabidopsis *overexpressing ACL

A. Weight plotted as shoot-root ratio for the wild type (Columbia ecotype (Col)) and for the transgenic plants overexpressing the two subunits of ATP citrate lyase (ACLa_ACLb). B. pH of the one-week old hydroponic solution with (black) or without (grey) phosphate in the medium. The error bars represent the standard deviation.

HPLC analysis of organic acids and phenolic compounds in Arabidopsis thaliana

By observing the different spectra of plants grown with in P sufficient conditions, five main compounds appeared, corresponding to malate (RT = 8.6), fumarate (RT = 15.5), citrate (RT= 7.2) and the two phenolics with retention times of 11.5 and 20 minutes (see Figure 21 Ai for the secretion and 21 Aii for the internal contents). Slight differences could be observed between the wild type and the two ACL overexpressing lines (44.2.2 and 44.3.9), with higher peak values for the internal contents of the ACL line 44.2.2.

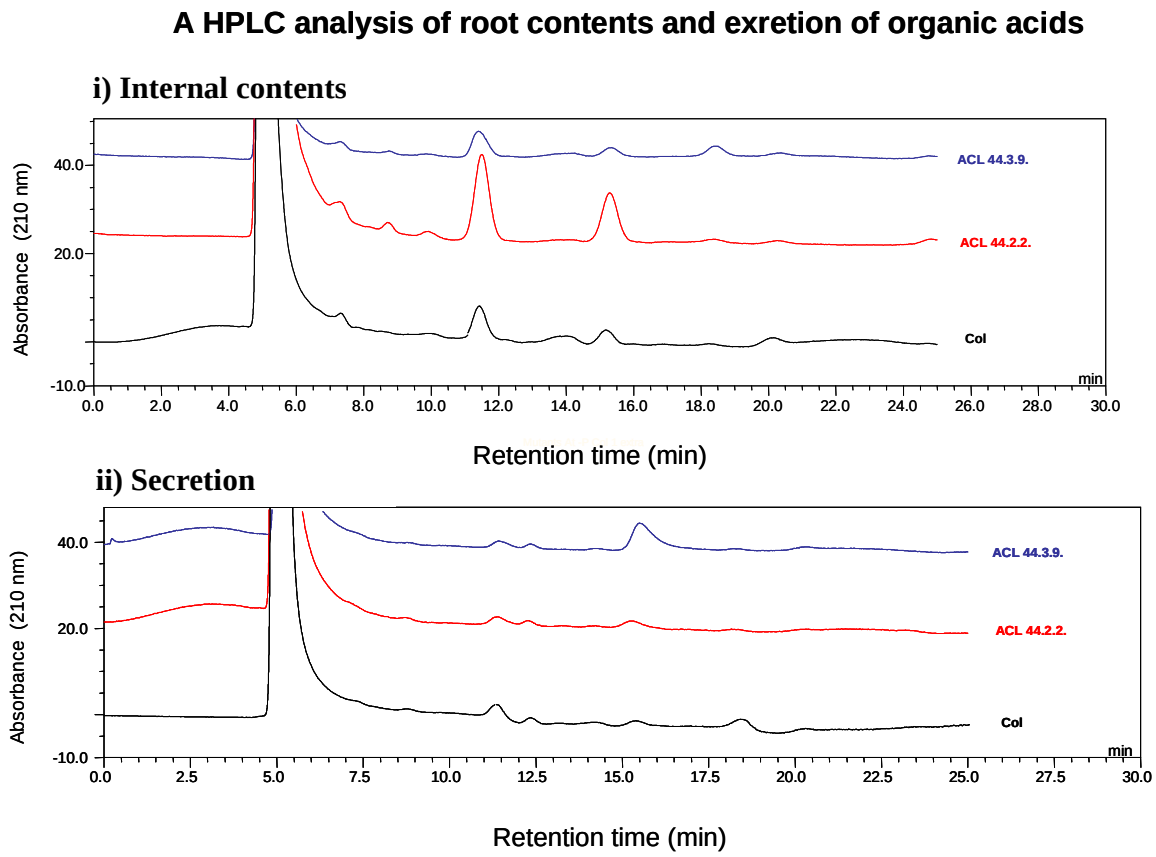


Figure 21: Organic acid secretion and contents in two lines of ACL overexpressing Arabidopsis thaliana

A. HPLC analysis of internal (i) and external (ii) organic acids in the presence of phosphate.

In general, the absence of phosphate increased the amounts of compounds present in the different lines and surprisingly, the differences observed were higher in the internal contents than in secretion (Figure 21 B).

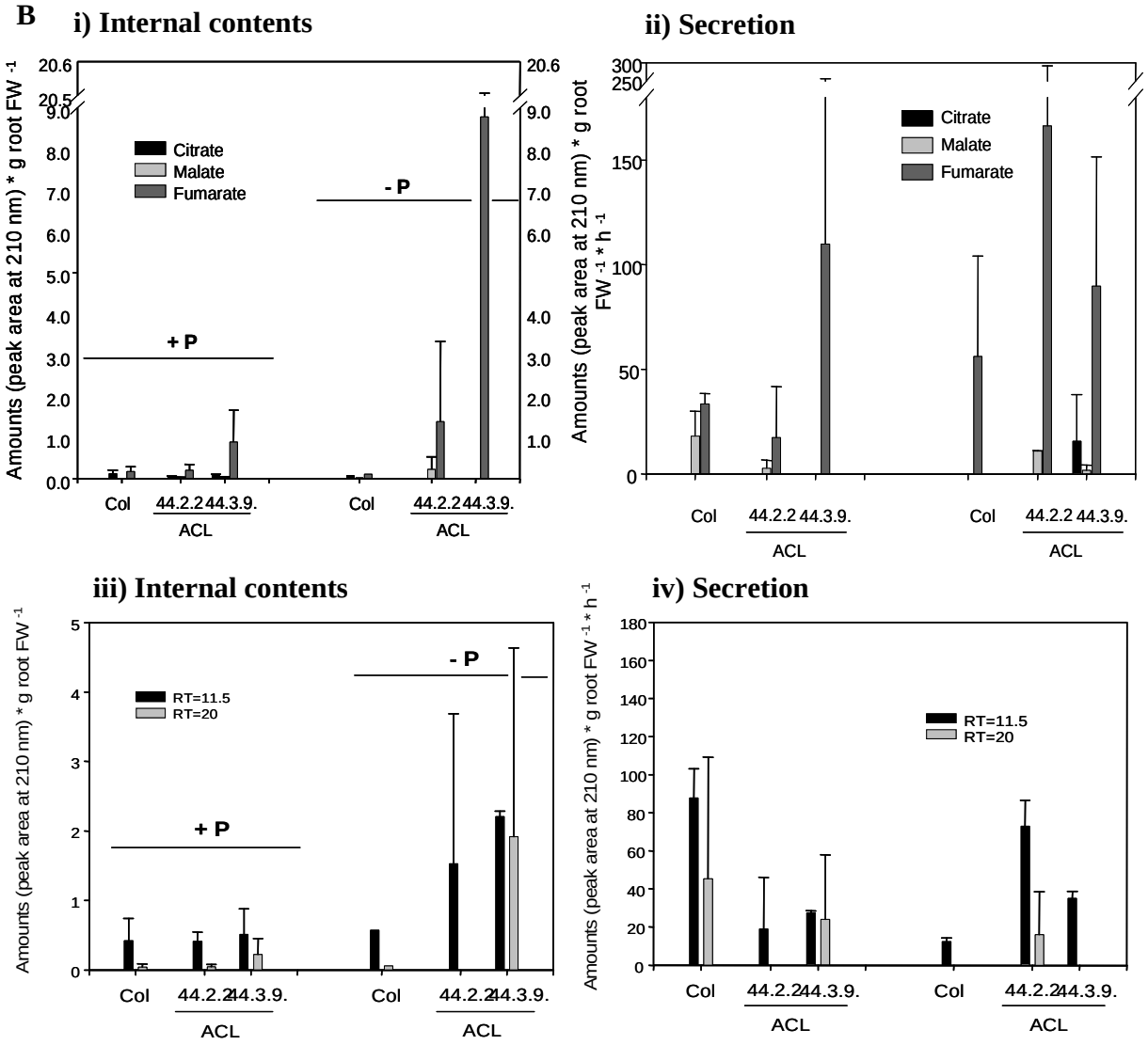


Figure 21 (cont.): Organic acid secretion and contents in two lines of Arabidopsis thaliana overexpressing ACL.

B. Quantification of the main compounds with (+ P) and without (- P) phosphate for the organic acids secreted (internal contents (i) and secretion (ii)) and phenolic compounds (internal contents (iii) and secretion (iv))

Quantification of the compounds present in and secreted from the roots allowed following observations: First, citrate was only secreted from the overexpressing ACL

Arabidopsis line 44.3.9 in absence of phosphate. In root tissues, however, low amounts were present in all lines in presence of phosphate but no citrate could be detected in P deficient ACL overexpressing lines. Secondly, for malate, we observed an effect of phosphate nutrition: with phosphate supply, the ACL overexpressing lines secreted lower amounts of malate than wild type plants, whereas in P deficient conditions, it was the opposite. Thirdly, fumarate was the compound present in highest amounts. In presence of phosphate, no differences were observed between the lines. In absence of phosphate, higher amounts of fumarate where observed in the overexpressing ACL lines. Finally, for the phenolic compounds, no clear trends were observed in P sufficient conditions, but higher amounts were observed in the absence of phosphate in the overexpressing ACL lines, suggesting a possible involvement of the acetyl-CoA produced by ACL activity in the biosynthesis of phenolic compounds.

2.2.3.5. Discussion

Localization of pCAMBIA-ACLa-(ACLb-GFP)

The localization of ACL has been studied in different plants and depending on the plant studied, different localizations for ACL were found. On the one hand, in developing seeds of *Brassica napus*, Ratledge and colleagues (1997) found that most of the ACL activity was localized in chloroplasts and they established a correlation between ACL activity and the rate of lipid synthesis. On the other hand, in *Pisum sativum* (Kaethner and Rees, 1985) or *Cyanophora paradoxa* (Ma et al., 2001b), ACL activity was exclusively localized in the cytosol, which is coherent with the primary role of this enzyme, namely the generation of cytosolic acetyl-CoA (Nikolau et al., 2000). Fatland et al. (2002) localized ACL in the cytosol of *Arabidopsis*. In our study, we localized the ACL from white lupin expressed in *Arabidopsis* using a genetic fusion with an enhanced GFP version (GFP6; Humair et al., 2001). We observed fluorescence at the periphery of the cells. As young roots have small vacuoles, a vacuolar or cytosolic localization is unlikely. Therefore, the fluorescence suggests a plasma membrane localization. To our knowledge, this is the first time that this localization is suggested. One possibility to explain this localization could be that the protein is cytoplasmic but may be partially attached to the plasma membrane or plasma membrane proteins. Another possibility would be that the fluorescence observed is not due to GFP but to cell wall components. Confirmation of the plasma membrane localization using Western blot analysis and purified plasma membrane would be required to see if ACL is indeed attached to the plasma membrane.

Physiological analysis of transgenic tobacco and Arabidopsis

No significant effect could be observed upon expression of the white lupin ACL gene in tobacco or *Arabidopsis thaliana* for the different physiologic parameters measured (shoot to root ratio, ACL specific activity or pH acidification). Moreover, the transformation of tobacco with an empty vector mimicked the trends observed with ACL. This suggests a lack of specificity in the effects observed in the overexpressing ACL tobacco, like higher

biomass and higher ACL activity. However, it is not the first report of such a lack of effects observed when an enzyme involved in organic acid synthesis is overexpressed. Delhaize et al. (2001) introduced a citrate synthase of *Pseudomonas aeruginosa* into tobacco and found no increase in the citrate synthase activity or enhanced citrate accumulation or efflux, even if its expression level was 100-fold higher in the transgenic tobacco. They postulated an incorrect folding or the formation of inactive aggregates in the host plant resulting in an inactive enzyme. A similar problem may have occurred in our case, but other factors could also account for the lack of difference between wild-type and transformed plants: first, the fact that ACL is essential for normal growth and development has been observed by Fatland et al. (2005), who found that plants with even moderately reduced ACL activity had a complex, bonsai phenotype due to the decrease of ACL-derived acetyl-CoA. The levels of organic acids needed by the plant often are already reached by the native enzymes and the activity of introduced enzymes often is regulated at the posttranscriptional level, which results in no difference in organic acid levels between wild-type and overexpressing plants, even though the expression levels of the corresponding enzyme might be much higher in the overexpressing plants. Secondly, it is a well-known fact that introduced overexpressed genes, independently of the function of the encoded enzyme, often are subject to internal silencing mechanisms (Meins, 1989 and Palauqui et al., 1997) and this explanation could also account for the "inverse" results obtained, namely the enhanced citrate production and secretion in ACL-overexpressing plants.

Organic acid secretion by roots of transgenic Nicotiana tabacum *and* Arabidopsis thaliana

In tobacco, four main compounds were detected in tissues and in the root exudates corresponding to citrate, fumarate and two unidentified phenolic compounds. We did not observe any major difference between the different lines except the presence of citrate in the ACL overexpressing tobacco. Citrate is known to be involved in aluminium tolerance mechanism in maize root (Piñeros et al., 2002). No malate was observed intra- or extracellularly in tobacco, in contrast to the situation in *Arabidopsis thaliana*, indicating

that malate exudates are very low. The lack of increase in organic acids accumulation or efflux in presence of aluminium (except for fumarate, which is not known to be involved in tolerance mechanism to heavy metals) is somewhat surprising due to the large bibliography showing the role of organic acids (and in particular malate, citrate and oxaloacetate) in aluminium tolerance in *Arabidopsis thaliana*, maize or triticale (Gaume et al., 2001b; Ligada et al., 2004 or Ma et al., 2000, 2001). Only Larsen et al. (1998) found that in *Arabidopsis*, the aluminium resistant *alr-104* mutant showed no enhanced release of malate or citrate but an enhanced acidification of the rhizosphere. However, in our case, we did not observed any difference in the pH of the media between the wild type and the transgenic plants.

In *Arabidopsis thaliana*, in addition to the four main compounds present in tobacco, malate was also present in little, but detectable amounts. We observed in all the cases high levels of organic acids in the extracellular environment meaning that these organic compounds (citrate, malate and fumarate) were secreted from the roots. The mechanisms responsible for this secretion are yet not fully understood. For the moment, only an aluminium activated malate channel has been described (Sasaki et al., 2004). In addition, Zhang et al. (2004) recently characterized two anion conductances in white lupin protoplasts responsible for citrate (and malate) efflux. In P deficient conditions, we observed higher amounts of malate and citrate intra- and extracellularly in the overexpressing ACL lines. This confirms the role played by the organic acids in phosphate deficient conditions, where they take part in acidification and solubilization of phosphate (Neumann and Römheld, 1999). Moreover, in phosphate deficient plants, malate was only present in the roots and exudates of the ACL overexpressing lines, supporting the relationship between the ACL activity and the malate exudation observed by Langlade et al. (2002) in maize and lupin roots. However, we were able to detect only very low amounts of organic acids, probably due to the non optimal hydroponic culture system, in which *Arabidopsis* plants produced only little root biomass. Thus, our results can be viewed as a preliminary support of the hypothesis that ACL activity might lead to an enhanced malate biosynthesis and secretion through production of its precursor oxaloacetate. In order to get a conclusive proof of this link, we still need to have a

significant effect on ACL activity in the transgenic lines, concomitantly to an enhanced secretion of malate. As for the role of ACL in providing the acetyl-CoA needed for phenolic biosynthesis, which would partly explain the higher synthesis and secretion of isoflavonoids occurring at the juvenile stage of cluster roots, our results indicate a possible effect since more phenolic compounds were secreted from the roots of ACL overexpressing plants than from the wild type plants. However, these also are preliminary indications and would require further experiments to obtain conclusive proofs.

2.2.4. Synthesis

This chapter dealing with the role ATP citrate lyase in organic acid metabolism was divided into two parts: first, the characterization of the white lupin ACL, which was then used for transformation, and secondly, the *Agrobacterium*-mediated transformation of candidate plants.

Langlade and collaborators (2002) found that the white lupin ACL is constituted of two subunits both required for enzyme activity and corresponding to the N- and C-terminals of the mammalian, as already found by Fatland et al. **(2002) in *Arabidopsis thaliana*. Furthermore, a putative novel function of ACL consisting in the switch between malate and citrate secretion in the developing cluster roots was presented. This is the first report suggesting the involvement of ACL in organic acid metabolism.**

Our data on *Arabidopsis* plants overexpressing the lupin ACL support this possible new role in plants, since malate internal contents and secretion were increased as compared to wild-type *Arabidopsis*. Moreover, higher amounts of two phenolic compounds were also observed in the ACL overexpressing plants, suggesting that in juvenile cluster roots, ACL activity might be involved in the biosynthesis of isoflavonoids as well. However, as mentioned previously, additional work is needed to confirm these preliminary results, since in our experiments, only two lines were studied, little amounts of organic acids were retrieved and the ACL activity showed no significant increase in the transgenic lines.

2.2.5. Discussion – Outlook

Taken all together, these results indicate on the one hand, that white lupin ACL is not only required for the supply of the cytosolic acetyl-CoA, as generally believed, but that it is also involved in the production of malate in white lupin cluster roots. This was deduced from a strong correlation between ACL activity and malate secretion in different developmental stages of white lupin cluster roots. At the juvenile stage, ACL activity is highest, and so are the amounts of malate secreted. Moreover, isoflavonoid secretion is also highest at this stage of development. At the mature stage, ACL activity is downregulated, malate secretion decreases, while citrate secretion concomitantly increases and isoflavonoid secretion also decreases. However, when overexpressing ACL in tobacco and *Arabidopsis*, no general increase was observed and only a slight one in the case of *Arabidopsis* grown in P deficient conditions. But this should not lead to reject the hypothesis that ACL is involved in organic acid metabolism. Rather should one improve the experimental concept used to test this hypothesis: when considering the high variability between replicates and the generally very low amounts of organic acids recovered from roots of plants grown in our hydroponic system, it is clear that these experiments do not allow to conclude in a final manner about the effects or the lack of effects of overexpressed ACL on the amounts of organic acids secreted. To obtain more convincing results, the following points should be considered: i) working with more replicates, not only within transgenic lines, but also with more different lines, ii) improving the hydroponic culture system in order to increase the root biomass, which might lead to a reduction of random effects and a better reproducibility of the analyses and finally iii) carefully selecting the promoter and favor a root-specific and inducible promoter, which may prevent or at least reduce the potential silencing reaction from the plant.

2.3. PHOSPHATE ACQUISITION BY WHITE LUPIN DURING A ONE YEAR CULTIVATION STUDY IN MICROCOSMS

2.3.1. Introduction – Context of the study

This work was conducted within the frame of the global project NCCR (national center of competence in research) number 4, devoted to the study of plant nutrition under stress conditions. The experimental setup was designed to meet the requirements of plant physiologists, soil biologists and microbiologists, as well as geologists. Most of the work was performed by Dr. C. Le Bayon, who was in charge of the project coordination, but also designed the experimental setup and did most of the analyses herself. The project was organized with the aim of investigating root induced changes at several levels of the rock-plant gradient and included two types of nutrition stresses: phosphate deficiency on the one hand and heavy metal toxicity on the other hand. Two groups of plant physiologists were focusing on one of these questions, our group (Prof. E. Martinoia and I) dealing with P deficiency and Prof. U. Feller with his PhD student V. Page, from the University of Bern, dealing with transport of heavy metals within the plant. Soil biologists (Prof. J.-M. Gobat and Dr. C. Le Bayon), microbiologists (Prof. M. Aragno and I) and geologists (Prof. K. Föllmi and Dr. V. Matera) completed the group.

The aim was to monitor phosphorus and heavy metal dynamics in the plant soil system, using white lupin and wheat as model plants. The original idea was to compare the behavior of these two species with respect to phosphate uptake and phosphorus dynamics. The reasons which led to the choice of white lupin and wheat were more political than scientific, white lupin being the P deficiency model plant of Prof. E. Martinoia and me and wheat being the heavy metal model plant of Prof. U. Feller and V. Page. Comparing a non-mycorrhized, cluster root forming plant with a mycorrhized plant surely could be very interesting, if the two plants would be closer to each other than it was the case here. Comparing *Medicago sativa* and *Lupinus albus*, for example, would have been more interesting. Moreover, a prerequisite for the proper comparison of the "mycorrhiza

strategy" and the "cluster root strategy" in terms of phosphate acquisition efficiency would be to verify that wheat indeed was mycorrhized in our experimental conditions… Since this was not done, we thought it wiser to treat the results obtained for the two plants separately and we present in the following chapter only the experiments performed with white lupin and investigating phosphorus dynamics. The part dealing with phosphorus dynamics in wheat, as well as the experiments analyzing heavy metal transport in wheat or in white lupin will be published separately. In addition to this assessment of P and heavy metal dynamics, DNA and RNA were extracted from these different samples, in lupin as well as in wheat, and molecular fingerprinting techniques based on DNA and RNA polymorphism were used to monitor changes in bacterial communities occurring along a spatial (root proximity) and temporal (incubation time) gradient. The data analysis on theses results is still ongoing and they will be published separately.

In the part presented in the following chapter (2.3.2.), we cultivated white lupin in microcosms for one year (replanting after 8 weeks of growth) and analyzed the P uptake by the plant, as well as the mineralization processes occurring at different root proximity levels. We investigated the root induced changes in P organic and inorganic pools in the rhizosphere soil of cluster and non cluster roots and compared these rhizosphere samples with two kinds of bulk soil samples: one which was harvested in the central element of the microcosms, i.e. where plants had produced roots and the other which was collected in side-arms of the microcosms, where the absence of root was guaranteed by a nylon mesh separation between the central tube and the side arms. Analyses included total phosphorus, extractable phosphorus (bioavailable phosphate), acid and alkaline phosphatase activities and amounts of fumarate, citrate and malate, identified as the major organic acids in white lupin rhizosphere.

2.3.2. Soil phosphorus uptake by continuously cropped *Lupinus albus*. A new microcosm design

To be submitted to Plant and Soil

By ***Claire Le Bayon****, Laure Weisskopf, Enrico Martinoia, Jan Jansa, Emmanuel Frossard, Felix Keller , Karl Föllmi and Jean-Michel Gobat*

2.3.2.1. Abstract

In nature, plants often have to grow on soils where phosphate availability is low and they have evolved several mechanisms to cope with this problem. When grown in soils with sparingly available phosphate, white lupin forms special root structures, called cluster roots, which secrete large amounts of organic acids and concomitantly acidify the rhizosphere. Many studies dealing with the understanding of this P acquisition strategy have been performed in short time experiments either in hydroponic cultures or in small microcosm designs with sand or sand: soil mixtures. In the present study, we aimed at designing an experimental setup which would come nearer to the natural field conditions. We performed a one-year experiment on large microcosms containing a total of 7 kg of soil and allowing separation of rhizosphere soil and bulk soil. We planted six successive generations of lupins and monitored P dynamics along a spatial-temporal gradient. We investigated the P uptake of plants, the organic P desorption, the acid and alkaline phosphatase activities and the organic acid contents in different soil samples, comparing rhizosphere soil of cluster and non cluster roots as well as bulk soil samples. A total shoot biomass of 55.69 ± 1.51 g (d.w.) y^{-1} was produced and P uptake reached 220.59 ± 5.99 mg y^{-1}. More P was desorbed from the rhizosphere soil of cluster roots than from the rhizosphere soil of non cluster roots or from the bulk soil ($P < 0.05$). For both acid and alkaline phosphatases, the rhizosphere soil samples associated to cluster (RSC) and non cluster roots (RSNC) of white lupin showed a generally higher activity than the bulk soil samples. The acid phosphatase activity in the RSC

stayed higher than in RSNC over the incubation time. In addition, a strong negative correlation was found between the acid phosphatase activity and the organic phosphorus (Spearman's correlation, rho = -0.93, P = 0.02). Regarding organic acids, fumarate was the most abundant organic acid, followed by malate and citrate. Citrate was only present in detectable amounts in RSC. In contrast, malate and fumarate were present in significantly higher amounts in RSNC (P<0.05), while almost no organic acids could be detected in the bulk soil. Our results demonstrate that over a one year cultivation period, important desorption processes took place in the rhizosphere of white lupin and that most of this desorption, as well as most of the phosphatase activity and organic acid secretion occurred in the rhizosphere of cluster roots, while the same processes took place in non cluster roots, but to a much lesser extent.

2.3.2.2. Introduction

In the soils, phosphorus (P) availability is usually low. The two main forms of P are the precipitated inorganic phosphate, which forms complexes with Ca, Fe or Al and the phosphate covalently bound to organic molecules. The only form of P available to plants is the inorganic phosphate present in the soil solution, which rarely exceeds 10 μM (Schachtman et al., 1998). As a consequence, P deficiency is one of the major limitations for crop production in many tropical and sub-tropical agroecosystems (Fairhust et al., 1999). Moreover, it has been estimated that inexpensive sources of rock phosphate used as P fertilizers might be depleted very soon, in a time range of 60 to 80 years (Vance, 2001) and P deficiency problems would then no longer be restricted to tropical and subtropical agricultural context. To cope with this problem of low P availability in soils, plants have developed various strategies for efficient P acquisition, like i) extensive root formation, ii) association with mycorrhizal fungi (Abbott and Robson, 1982; Azaizeh et al., 1995; Khaliq and Sanders, 2000), iii) secretion of organic acids (Jones, 1998) and phosphatases (Gaume et al., 2001a), iv) increased expression of high-affinity phosphate transporters (Liu et al., 2001) or v) the development of specialized roots structures known as cluster roots (Purnell, 1960; Keerthisinghe et al., 1998; Lamont, 2003; Neumann and Martinoia, 2002; Shane and Lambers, *in press*).

White lupin (*Lupinus albus* L.) is the sole cluster root forming species of agricultural importance and is often considered as a model plant for studying the formation of cluster roots. Cluster roots can represent more than 60 % of the total root biomass (Keerthisinghe et al., 1998) and in comparison with non cluster roots, they can increase the surface area by a factor of over 140 times per length unit and the volume of soil explored by 288 times per length unit (Lamont, 2003). This large surface-volume ratio improves nutrient uptake as well as solubilization processes. Cluster roots are known to exude large amounts of low-molecular-weight organic anions, especially citrate, which enhances the availability of P to the plants (Gardner et al., 1982b, 1982c, 1983; Dinkelaker et al., 1989; Gerke et al., 1994). At the cellular level, Jones (1998) hypothesized two main routes of organic acid efflux from roots to the soil solution: i) a slow passive diffusion across the lipid

bilayer and, ii) an efflux through a plasma membrane channel protein. In white lupin, carboxylates are secreted in the unprotonated form through recently identified channels (Sasaki et al., 2004; Zhang et al., 2004), whereas acidification is due to the activation of the plasma membrane proton pump (Yan et al., 2002). Depending on the dissociation properties and number of the carboxylic groups, such organic anions may complex metal cations like Al^{3+}, Fe^{3+} in acid soils and/or Ca^{2+} and Mg^{2+} in alkaline soils. In this context, Gardner et al. (1983) suggested that ferric-hydroxyl-phosphate-citrate polymers could be formed and then diffuse to the root surface where reducing agents would liberate the phosphate. Alternatively, ligand exchange of phosphate by citrate can also occur (Gerke et al., 1994). Root clusters also secrete enzymes into the rhizosphere, especially acid phosphatases that hydrolyze organic phosphates (Gilbert et al., 1999; Wasaki et al., 1999, 2003). All these processes are involved in P mobilization and white lupin can thus take up P not only from the pool of phosphate normally available to plants, but also from the stable residual soil P fractions (Kamh et al., 1999) that cannot be utilized by other plants like soybean (Braum and Helmke, 1995).

Many studies have focused on P nutrition and part of them were conducted in hydroponic conditions (Hagström et al., 2001; Sas et al., 2001; Penazola et al., 2002; Wasaki et al., 2003) using the hydroponic system for a better understanding of the processes involved. The use of sand cultures (Liu et al., 2001) and mixtures of sand and soil (4:1, Cu et al., 2004; 1:1, Watt and Evans, 2003) constituted the next step, with a solid substrate but still quite far away from the field conditions. A further important progress was to work with a whole soil matrix, and this was performed using several experimental designs like rhizoboxes (Moritsuka et al., 2000), small pots filled with 450 to 800 g soil (Braume and Helmke, 1995) and plastic bags containing around 4 kg soil (Wouterlood et al., 2004). Larger experimental pots made of 10-cm diameter PVC pipe and 60 cm tall were used by Cavigelli and Thien (2003), and Bolland (1997) worked in the field on plots 1.4 m wide and 5 m long. Another important issue when studying plant nutrition is the length of the cultivation time. In most studies, short-time experiments were conducted (17 days, Moritsuka et al., 2000; 18 days, Gaume et al., 2001a; 35 days, Watt and Evans, 2003; 42

days, Cu et al., 2004), whereas only in few cases, longer time scales were used (from 57 to 64 days, Braume and Helmke, 1995; from 127 to 224 days, Bolland, 1997).

As a consequence, a sufficiently large design and an extensive duration of experiments are both needed to obtain a more ecological view of the plant nutrition process and to better extrapolate the results from hydroponics and small pots to the whole agroecosystem. In this context, the aim of our study was to conduct an experiment to better understand the P acquisition in relation to a spatial-temporal scale. For this purpose, we have developed in the present work a novel design based on the Starpot model (Jansa et al., 2003) which enabled us to work at different distances from the roots. In particular, the implementation of root-free compartments in our microcosms allowed a clear separation between soil adhering to the roots (rhizosphere soil) and the non-adhering distant soil (bulk soil). Since we focused on P nutrition, we used white lupin (*Lupinus albus* L.) as a model plant to investigate P uptake, changes in organic and inorganic pools of phosphate, as well as the activity of phosphatases and the secretion of organic acids during an incubation time of one year. In natural conditions, white lupin reaches the grain state after 224 days (Bolland, 1997); however, in our growth chamber, the flowering state was obtained after 60 days. Consequently, six successive generations of lupin were planted and P dynamics was investigated along a spatial-temporal gradient, by analyzing the different forms of phosphate, phosphatase activity and organic acids at different root proximities and incubation times.

2.3.2.3. Material and methods

Experimental design and growth conditions

The soil was collected from an agroecosystem regularly cultivated with wheat (Corcelles-Concise, VD, Switzerland) from 5 to 30 cm depth (Ap horizon) of the soil profile. This loamy soil (50 % sand, 30 % silt and 20 % clay), is a chromic luvisol (FAO) with the following characteristics: 1.3 % of carbon (organic carbon: 0.97 % and mineral carbon: 0.31 %), C/N: 11.8, pH $_{H2O}$: 7.8, pH $_{KCl}$: 7.02, a total phosphorus of 1407.8 (± 74.2) mg P

kg^{-1}. The soil was air-dried, sieved at 2 mm and remoistened to a water content of 20 % w:w prior to fill microcosms at a bulk density of 1.35 $g.cm^{-3}$. Microcosms were based on the Starpot's model (Jansa et al., 2003) and consisted of a central PVC tube (15 cm internal diameter and 35 cm height) and four small perpendicular tubes ("side arms", 4 cm diameter and 20 cm length). Four side arms were inserted into the central tube in order to respect the symmetry with the four plants sowed further (Figure 22). We used a baked clay system (Oil Dry Chem-Sorb WR24/18, Brenntag, Vitrolles, France; also provided by Maag Technics, Dübendorf, Switzerland) for the watering of the side arms. Root penetration from the central tube to the side arms was avoided by inserting a 25 µm-nylon mesh (SEFAR NITEX 03-25/19) between the side arms and the central tube. This system allows the separation of bulk soil and soil in contact with roots, but the root hairs can pass through the nylon mesh (diameter from 5 to 17 µm; Dittmer, 1949).

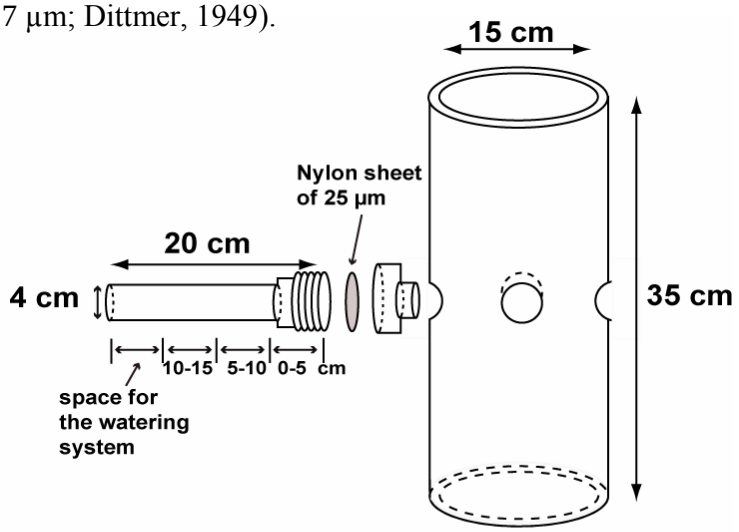


Figure 22: Microcosm

Scheme of a microcosm composed of a central PVC tube and four side arms. A nylon mesh of 25 µm is inserted between each arm and the central tube. Sites for soil collection in the arms are indicated.

All microcosms were kept in a growth chamber under controlled conditions with temperature of 24/20 °C, about 50 % humidity and a photoperiod of 16 to 8 hours (day/night, respectively) with a light intensity of 8000 lux. Seeds of white lupin (*Lupinus albus* L., cv. Amiga, Suedwestdeutsche Saatzucht, Rastatt, Germany) were incubated overnight in aerated deionised water before being placed in between filter paper sheets moistened with 0.2 mM $CaSO_4$ for four days in the dark and one day in the light. Four seeds were transplanted into the central tube of the microcosms. Microcosms for the two different treatments, with lupin (codified L) or without lupin (control, codified C), were

prepared in triplicates for eight sampling dates: 1, 2, 3, 4, 5, 6, 9 and 12 months. For watering, we used a nutrient solution without P containing (in mg l^{-1}): $Ca(NO_3)_2$, $4H_2O$ (118), K_2SO_4 (32.6), $MgSO_4$, $7H_2O$ (40), FeNaEDTA (3.67), KCl (0.93), H_3BO_3 (0.46), $MnSO_4$,H_2O (0.42), $CuSO_4$, $5H_2O$ (0.06), $ZnSO_4$, $7H_2O$ (0.072), $Mo_7O_{24}(NH_4)_6$ (0.93). Over the incubation time, no water leached at the bottom of the soil column.

Harvest and sampling

At the beginning of the lupin flowering state (after 60 days in our experimental conditions), all the shoot biomass (leaves, stems and the first flower) were harvested and pooled together. Immediately after harvesting, four new germinated seeds were planted in the central tube. At each of the eight sampling dates, three microcosms of each treatment (with and without lupin) were harvested. Shoots from the four plants were harvested, pooled per pot, and dried at 40°C to avoid carbon or nitrogen losses. Several soil samples were collected: from the central tube, roots were recovered (separately the cluster and non cluster roots) and bulk soil sample (not adhering to roots) were collected. Number of cluster and non cluster roots was quite similar at each sampling date, and all roots were linked together. This suggests that roots collected came from the upper alive plants, the older ones having been decomposed. Rhizosphere soil was gently shaken off the roots by hand and sieved through 1 mm mesh (undesirable fine roots found in the sieved soil were carefully removed) and collected. From the side arms, three soil samples were obtained with respect to the distance to the central tube: 0-5 cm, 5-10 cm and 10-15 cm. Samples from same distances in the four different side arms were pooled together. Samples from the central cylinder were labeled as follows: i) bulk soil: BS, ii) rhizosphere soil from cluster roots: RSC, iii) rhizosphere soil from non cluster roots: RSNC. So, on our root-soil gradient ranging from the immediate root vicinity to the most distant soil, six different soil samples were collected: the rhizosphere soil of cluster roots (RSC) and non cluster roots (RSNC), the bulk soil (BS) collected from the central cylinder and the three soil sections from the side arms, 0-5 cm, 5-10 cm and 10-15 cm. From each sample, one part was stored at -80 °C for organic acids analysis, while another part was stored at 4 °C for enzyme analyses.

Analytical procedures

Soil phosphatase activities

Analysis of phosphatase activity (phosphomonoesterase) was based on the release of p-nitrophenol after soil incubation with p-nitrophenyl phosphate in a modified universal buffer MUB (pH 6.5 for the assay of acid phosphatase activity, AcPA, and pH 11 for the assay of alkaline phosphatase activity, AkPA), as described by Tabatabai and Bremner (1969, for a review, see also Eivazi and Tabatabai, 1977). Acid phosphatases have been detected in animal, microbial and plant cells while alkaline enzymes have been found in microorganisms and animals (see the review by Alef and Nannipieri, 1995). Reaction mixture contained 1g soil to which 1 ml p-nitrophenyl phosphate and 0.25 ml of toluene were added. Following the incubation for 1 h at 37 °C, the soil solution was filtered (Whatman GF/C) and the absorbance of the filtrate was measured at 400 nm (Spectrophotometer Libra S12, Biochrom).

Phosphorus analyses in soil and plants

Total phosphorus in both soil and plant samples was colorimetrically measured following a Kjeldahl oxidation. One gram of soil or 0.5 g (d.w.) of plant material was put into a digestion tube (Büchi) with two glass balls, a Kjeldahl tablet (Merck) and 12 ml of H_2SO_4 96 N. After digestion at 360 °C during 2 h, samples were cooled and 60 ml of deionised water were added. After filtration (Schleicher and Schuell, 512 ½), P was determined at 880 nm using the molybdate acid procedure (Murphy and Riley, 1962). Organic phosphorus (P_{org}) was assessed by igniting soil samples (1g d.w.) in a muffle furnace at 550 °C for 1 h (modified from Anderson and Ingram, 1993). Both ignited and unignited samples were placed into propylene tubes and 50 ml of H_2SO_4 0.5 N were added. After shaking the tubes overnight, samples were filtered (Schleicher and Schuell, 512 ½) and P was determined as above. The difference in the acid extractable P of ignited and unignited samples gave a measure of the organic P. Extractable phosphorus by plants was determined according to Olsen et al. (1954) by shaking 2.5 g of soil with 60 ml of sodium

bicarbonate $NaHCO_3$ (0.5 N, pH 8.5) during 30 minutes. Phosphate was determined in the filtrate (Schleicher and Schuell, 512 ½) using the molybdate acid procedure (see above).

Organic acids in soils

Organic acids were extracted by shaking soil (approximately 0.75 g f.w. and 1.5 ml of sterilized water) for 30 minutes at 1400 rpm. Samples were then centrifuged for 5 minutes at 14000 rpm. The supernatant was filtered through 0.2 µm (Semadeni, syringe filters, 4 mm diameter) and 600 µl were dried at 50 °C in a speed-vac. Samples were resuspended in distilled water and 100 µl were injected into the HPLC system. An anion-exchange column (300 x 7.8 mm, 10 µm, H+ form, Benson, Nevada) was used to separate the different organic acids with an isocratic flow of H_2SO_4 20 mM at 0.7 ml min^{-1}. Separation was achieved in 20 minutes and absorbance was monitored at 210 nm. Calibration curves with standard organic acids were performed for quantification of the most abundant organic acids present in the analyzed samples.

Statistical analysis

Statistical analyses were performed using the SPLUS software, version 6.1 (Insighful Corporation, Seattle, USA). Before analyses, data were tested for normal distribution and homogeneity of variance (Kolmogorov-Smirnov tests). Then, two-factor ANOVAs were performed to test the microsite (rhizophere *vs.* non rhizosphere soil) and the incubation time on total P, Porg, Olsen-P, and organic acids in the different soil samples. Pair-wise comparisons were made with Tukey-Kramer HSD tests (interval of confidence: 95 %) and the Student's t test (organic acids, interval of confidence: 95 %).

2.3.2.4. Results

The variations in the phosphorus pools, phosphatase activities and organic acids over cultivation time are shown in Figures 23 to 26. For the sake of clarity and because no significant changes were observed between the intermediate locations in the root-soil gradient, only the extreme locations are shown: the rhizosphere soil of both cluster and non cluster roots and the most distant soil (10-15 cm). Figure 27 presents a focus after 12 months and shows all the different intermediate locations in the root-soil gradient, for bioavailable phosphorus, phosphatase activity and organic acids.

Phosphate uptake by lupin

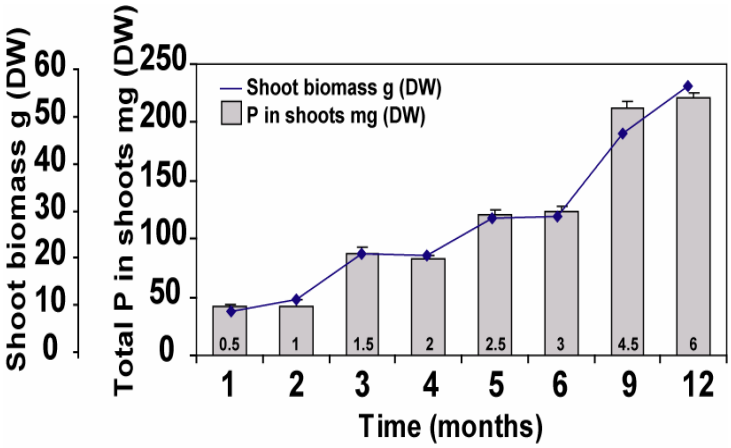


Figure 23: Phosphorus uptake

Total phosphorus (mg, histograms) in shoots of white lupin (four plants per pot) and total biomass of shoots (g dry weight, line) collected over the time. Total P from seeds was substracted. The number of lupin generations is indicated in

In all microcosms and for four plants, the total shoot produced in two months represented a mean of 9.66 ± 0.71 g (d.w.). Around 80 % of the biomass was reached after 1 month of growth, as illustrated by the plateaux observed at month 2, 4 and 6 (Figure 23, line). After substracting the P contents of seeds (2.93 ± 0.36 mgP g^{-1} seed (d.w.), phosphorus in shoots was calculated over cultivation time (Figure 23, histograms). A mean of 9.66 ± 0.71 g (d.w.) biomass was produced per microcosm (around 2.5 g (d.w.) $plant^{-1}$), representing a total biomass of 55.69 ± 1.51 g (d.w.) $year^{-1}$ and a total P uptake of 220.59 ± 5.99 mg $year^{-1}$. White lupins were able to take up and incorporate 2.62 % of the total P from the soil into the shoot biomass. To get more insights into the soil origin of this

uptaken phosphate, we investigated the organic and bioavailable pools of P along our spatio-temporal gradient.

Phosphorus in soil

For all soil fractions, the amounts of extractable P remained constant in the first 6 months and then decreased more and more till the end of the year (Figure 24 A). More P was desorbed from the rhizosphere soil of cluster roots (RSC) than from the rhizosphere soil of non cluster roots (RSNC) or from the bulk soil ($L_{10\text{-}15}$) ($P < 0.05$, Figure 24 A).

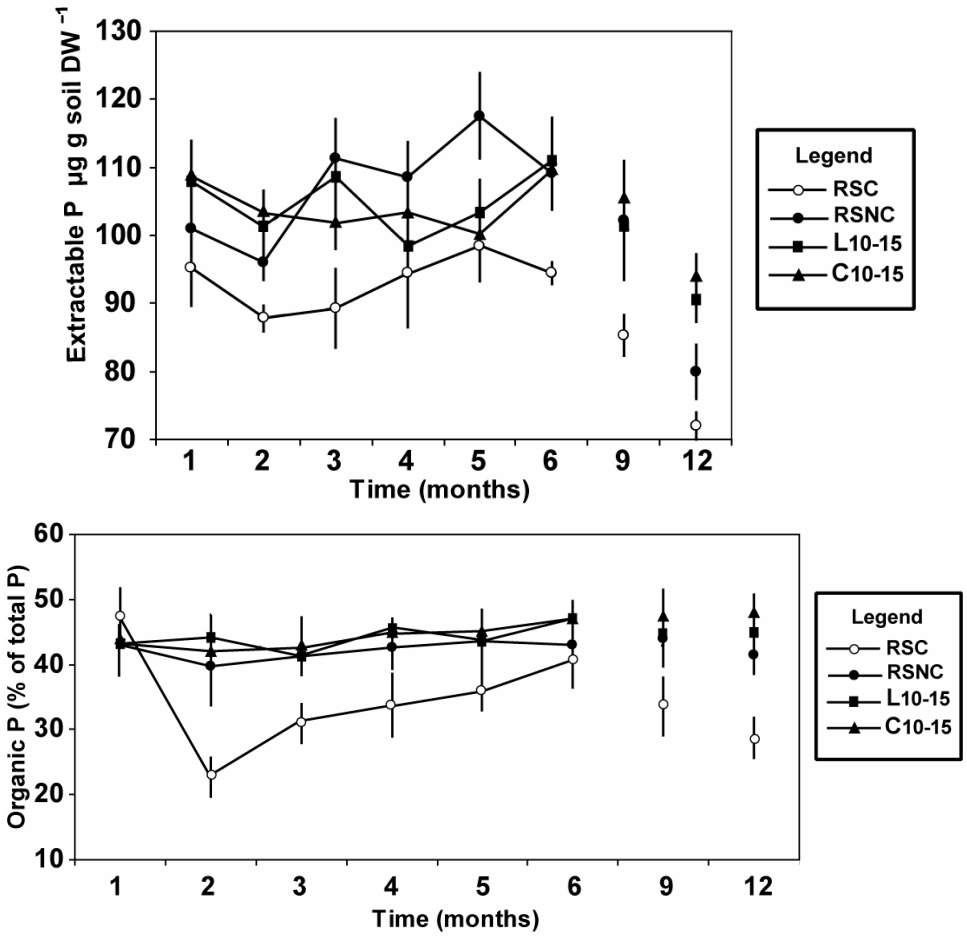


Figure 24: Phosphorus pools in different soil fractions

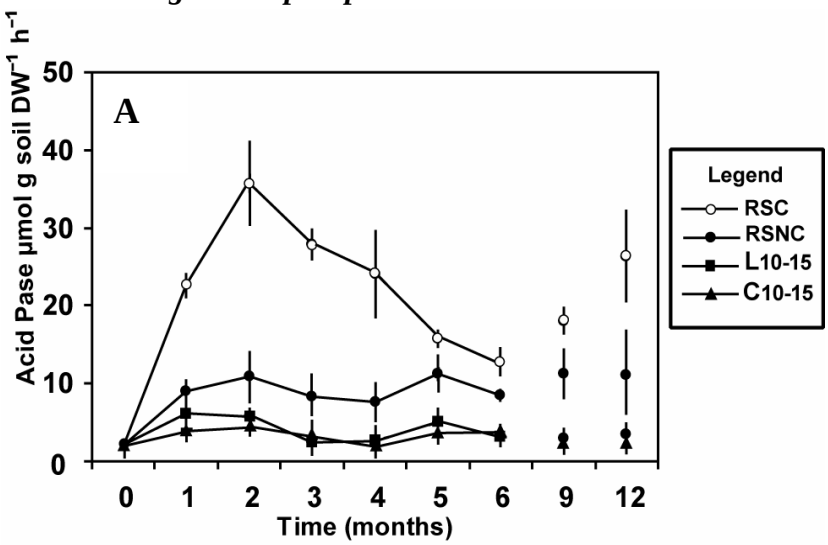
Soil bicarbonate extractable phosphorus (A, µg g soil dry weight^{-1}) and soil organic phosphorus (B, percentage of total P) over the time. $L_{10\text{-}15}$: most distant soil in the side arms, microcosms with lupin; $C_{10\text{-}15}$: most distant soil in the side arms, microcosms without lupin; RSC: rhizosphere soil from cluster roots; RSNC: rhizosphere soil from non cluster roots.

A decrease over time was also observed for organic phosphorus, but only in the rhizosphere soil of cluster roots (Figure 24 B, RSC). Except at month 1, RSC showed significantly lower levels of P_{org} ($P < 0.05$) compared to other samples, with a strong decrease at month 2 (22.7 % of the total P), month 9 (33.5 %) and 12 (28.8 %), suggesting that more P_{org} had been mineralized in the rhizosphere of cluster roots than in other soil samples. A temporal variability of extractable P in RSC was observed in parallel to P_{org} with a significant decrease at month 2 (1197 ± 12.01 mg kg^{-1}), month 3 (1275 ± 18.22 mg kg^{-1}), month 9 (1282 ± 8.20 mg kg^{-1}) and month 12 (1200 ± 9.50 mg kg^{-1}). In contrast to the differences observed between RSC and bulk soil samples, no significant difference between RSNC and bulk soil could be found, neither in P_{org} nor in extractable P, indicating that phosphate acquisition was mostly taking place in the rhizosphere of cluster roots. To investigate the possible solubilization and acquisition mechanisms underlying these changes in soil P pools, we analyzed phosphatase activities and organic acid levels.

Phosphatase activities

Figure 25 shows the activities of acid phosphatase (A) and alkaline phosphatase (B) in function of cultivation time and root proximity.

Figure 25: phosphatase activities



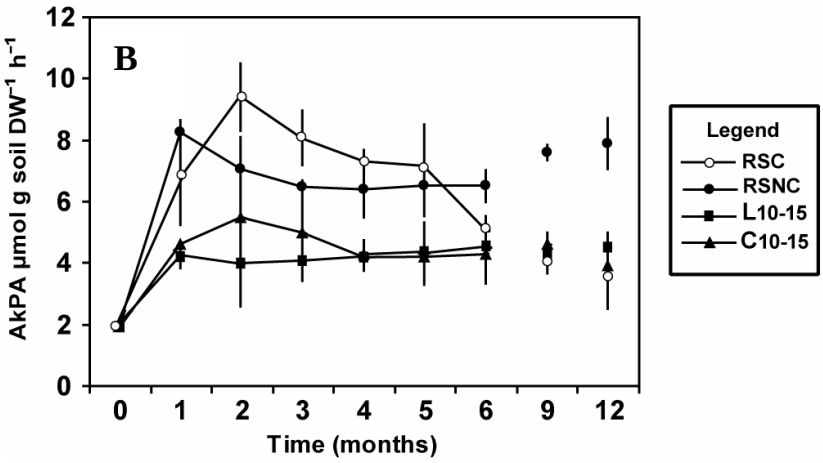


Figure 25: Phosphatase activity (µmol g soil dry weight^{-1}) in the soil fractions: A. Acid phosphatase activity (pH 6.5). B. alkaline phosphatase activity (pH 11). $L_{10\text{-}15}$: most distant soil in the side arms, microcosms with lupin; $C_{10\text{-}15}$: most distant soil in the side arms, microcosms without lupin; RSC: rhizosphere soil from cluster roots; RSNC: rhizosphere soil from non cluster roots.

For both enzymes, the rhizosphere soil samples associated to cluster (RSC) and non cluster roots (RSNC) showed a generally higher activity than the bulk soil samples of lupin microcosms ($L_{10\text{-}15}$) or of unplanted microcosms ($C_{10\text{-}15}$), clearly reflecting the influence of root proximity. The soil associated with cluster roots showed an enhanced acid phosphatase activity (Figure 25 A, RSC) compared with the soil associated with non cluster roots (RSNC) ($P < 0.05$). After two months of incubation, the acid phosphatase activity in the RSC reached a peak of 35.6 µmol g^{-1} h^{-1}, *i.e.* 3.5 times higher than in RSNC (10.8 µmol g^{-1} h^{-1}) and despite a high variability over time, the acid phosphatase activity stayed higher in RSC than in RSNC till the end of the experiment. The activity of alkaline phosphatase was generally low (from 4 to 6.4 µmol g^{-1} h^{-1}) but still a significant difference between rhizosphere soil and bulk soil fractions could be observed, especially in the rhizosphere soil of non cluster roots (RSNC) ($P < 0.05$) and in the rhizosphere of cluster roots (RSC) during the first 6 months.

As expected, there was a strong correlation between the acid phosphatase activity (Figure 25 A) and the organic phosphorus (Figure 24 B). The two variables were negatively correlated according to the Spearman's correlation (rho = -0.93, P = 0.02). This

correlation fits into the known hydrolysis mechanism of the organic pool and indicates that phosphatases may well account, at least partially, for the phosphate acquisition of white lupin, as shown in Figure 23. In addition to P_{org} which represents less than 50 % (Figure 24 B) of the total P present in our plant-soil system, other mechanisms devoted to the acquisition of phosphate must also play an important role in P nutrition. Since white lupin is known to secrete high amounts of organic acids, we determined organic acid contents in the different soil fractions.

Major organic acids

Figure 26 shows the major organic acids analyzed in the various soil fractions at the different sampling dates. Fumarate was the most abundant organic acid, followed by malate and citrate. Traces of other organic acids, like acetate or succinate, were also found in various soil samples. There was a high variability over time, especially in the rhizosphere soil samples. High amounts were found both after 2 months (first lupin generation) and after 12 months (sixth lupin generation) again.

In the rhizosphere soil of non cluster roots, a general decrease in organic acids could be observed at the 9^{th} month sampling date, which corresponds to the only case where lupins were four weeks old, instead of eight. Overall, significantly higher amounts of the three major organic acids were found in lupin than in the control pots ($P < 0.05$, data not shown). Citrate was much more abundant in RSC than in RSNC. In contrast, malate and fumarate were present in significantly higher amounts in RSNC ($P < 0.05$). As expected, almost no organic acids could be detected in the bulk soil or in the 10-15 cm section of the microcosm side-arms, except for little amounts of fumarate detected at some sampling dates. However, fumarate was also found in control pots (data not shown), indicating that its presence cannot be directly related to the root secretion activity.

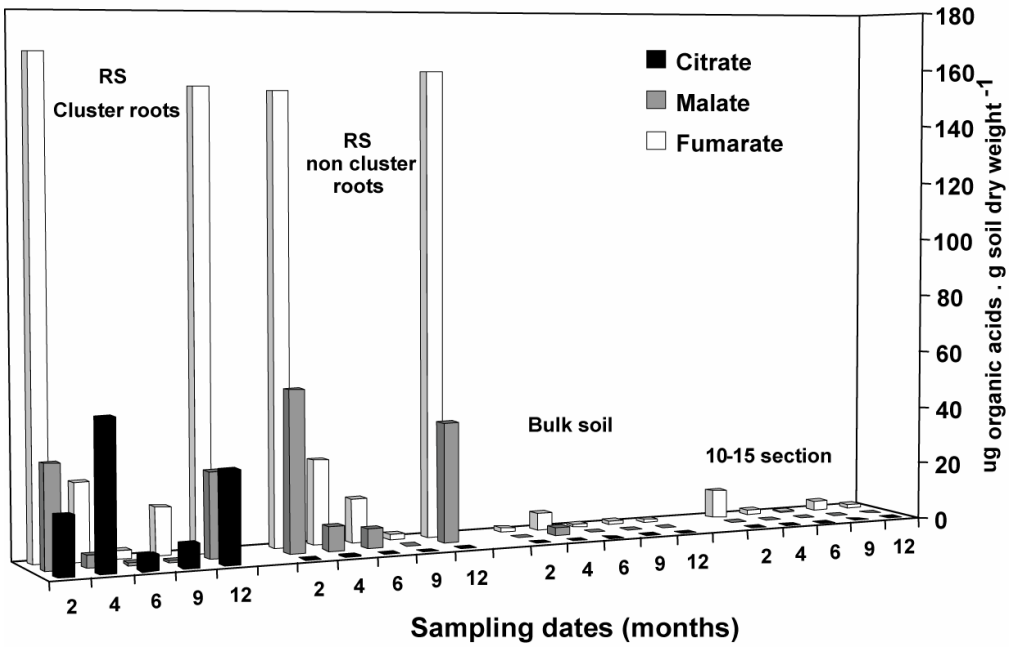


Figure 26: Amounts of carboxylates in the different soil samples

Amounts of citrate (black), malate (grey) and fumarate (white) in the different lupin soil fractions in µg. g soil dry weight^{-1}. RSC: rhizosphere soil of cluster roots; RSNC: rhizosphere soil of non cluster roots; BS: bulk soil (from the central cylinder of microcosms) and 10-15: most distant soil (from the adjacent tubes). Organic acids were separated by HPLC on an anion-exchange column and amounts were calculated from the peak areas based on calibration curves of the corresponding standards. Bars represent means of three replicates.

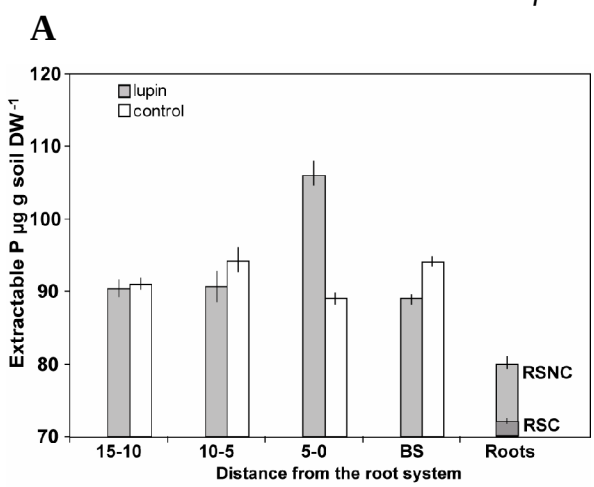
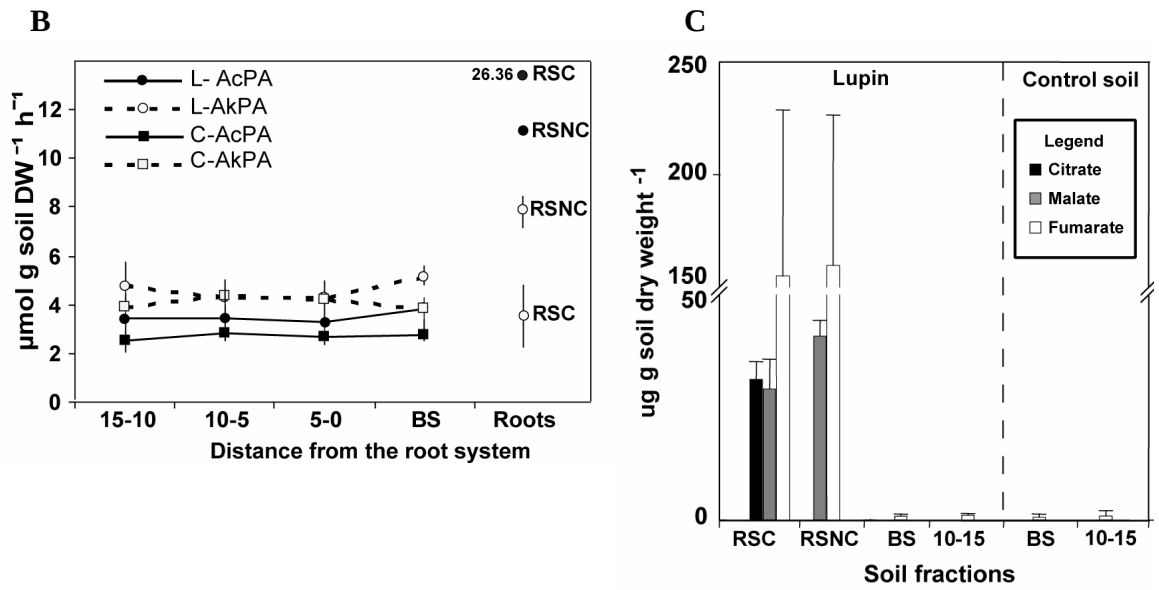


Figure 27 : Situation after one year
A. Soil bicarbonate extractable phosphorus

Figure 27 describes the situation after one year and shows the results for all locations along the root-soil gradient. In the three parameters analyzed, extractable P (Figure 27 A), phosphatase activities (Figure 27 B) and organic acids (Figure 27 C), no significant difference could be observed between the different soil distances, from the bulk soil collected in the central tube to

the last section of the side-arms, 10-15 cm, with the exception of the fraction 5-0, which showed higher amounts of extractable P in comparison with all other samples. For all three parameters (Figure 27 A, B and C), a clear rhizosphere effect could be shown for soil samples associated with cluster and non cluster roots.

Figure 27 (cont.): Situation after one year
B. Phosphatase activity. C. Organic acids after one year of cultivation.



A: Soil bicarbonate extractable phosphorus. Grey and white histograms for lupin and control microcosms, respectively; 0-5, 5-10, 10-15 cm: soil distant in the side arms; BS: bulk soil (from the central cylinder of microcosms); RSC: rhizosphere soil from cluster roots; RSNC: rhizosphere soil from non cluster roots

B: Acid (AcPA) and alkaline (AkPA) phosphatase activities in µmol g soil dry weight^{-1} h^{-1} in microcosms with and without lupin (L and C, respectively). 0-5, 5-10, 10-15 cm: soil distant in the side arms; BS: bulk soil (from the central cylinder of microcosms); RSC: rhizosphere soil from cluster roots; RSNC: rhizosphere soil from non cluster roots.

C: Amounts of citrate (black), malate (grey) and fumarate (white) in the different lupin and control soil fractions in µg. g soil dry weight^{-1}. RSC: rhizosphere soil of cluster roots; RSNC: rhizosphere soil of non cluster roots; BS: bulk soil (from the central cylinder of microcosms) and 10-15: most distant soil (from the adjacent tubes). Organic acids were separated by HPLC on an anion-exchange column and amounts were calculated from the peak areas based on calibration curves of the corresponding standards. Bars represent means of three replicates.

2.3.2.5. Discussion

The present study was conducted to monitor the phosphorus dynamics (sorption/desorption and acquisition by plants) in an experimental setup based on the Starpot model (Jansa et al., 2003) and which enabled us to come a step nearer to the natural field conditions. The originality of this design was the implementation of root-free compartments in our microcosms allowing to define three levels of root proximity, i) rhizosphere soil, ii) bulk soil (i.e. non adhering to roots) from the central compartment of microcosms and iii) bulk soil from the side-arms, where a complete absence of roots was guaranteed. We followed the changes in P uptake, extractable and organic P pools, phosphatase activities as well as organic acids during one year of cultivation and we could show a clear spatio-temporal evolution of most of the measured variables.

Shoot biomass reached 55.69 ± 1.51 g (d.w.) per microcosm at the end of the year. Despite the supply of iron and zinc through the nutrient solution and despite the relatively high amounts of bicarbonate extractable P in the original soil (108.8± 0.97 mg kg^{-1}), white lupins produced a lot of cluster roots, especially at the end of the one-year experiment. Our results show that P-adequate plants also form cluster roots as previously demonstrated by Shen et al. (2003) who support the hypothesis that a partial depletion of available P over time could have induced P deficiency and enhanced formation of cluster roots. In our study, the low amounts of organic P and bicarbonate extractable P, and conversely the high phosphatases activities and the great release of organic acids near the roots after a year confirmed this hypothesis.

Over the incubation time, there was a clear effect of cultivation time, especially in the rhizosphere soil samples. Particularly after month 2 and 12, an efficient P acquisition mechanism is reflected by the very low $NaHCO_3$ extractable phosphate levels left around the roots of white lupin, and especially around cluster roots (Figure 24 A and 27 A). The phosphatase activities (Figure 25 and 27 B), as well as the high amounts of organic acids (Figure 26 and 27c) revealed a complementary strategy to acquire phosphate from both

the organic and the inorganic pools. Thus, without addition of exogenous P, white lupins could still find some phosphate in the soil to take up till the end of the experiment.

We found that, in addition to the well-known secretion of organic acids, which was clearly demonstrated previously in hydroponic systems and confirmed in our study, phosphatases also played a major role in P acquisition. Again, we were able to confirm in an experimental situation which comes nearer to the real field conditions, the findings previously discovered and studied in hydroponic cultures (Game et al., 2001a; Washakie et al., 2003). Phosphatase activities were higher in the rhizosphere soil than in the bulk soil. In the rhizosphere of cluster roots, the high activity of acid phosphatase (AcPA) was strongly correlated with the amount of organic phosphorus over the incubation time. This suggests an intense hydrolysis of organic P over the year, with a Porg decreasing from 47 % to 29 % of total P (Figure 24 B) resulting in a considerable release of available P in the vicinity of cluster roots. The first peak of AcPA after two months of incubation (Figure 25 A) could be due either to a generally high secretion of root exudates including phosphatases, or to an intense microbial growth in response to these root exudates, and the production by these microbes of phosphatases. This particular behavior at the beginning of the experiment could be due to a necessary adjustment of the experimental system, since air-drying, sieving and remoistening the soil had certainly disturbed the microflora and micro fauna living in the soil. This was confirmed by an additional measure of urease activity that was the strongest at month 2 (data not showed). A minimum of time-resilience seems thus to be required to reach an equilibrated system. The high enzyme activity observed after twelve months may reflect an actual need of P for the white lupin growth. We observed also the presence of AcPA in RSCN, assessing a significant hydrolysis of P in the proximity of non cluster roots over the time. This is confirmed by Wasaki et al. (1999, 2003) who showed that not only cluster roots but the whole root system could secrete acid phosphatase. These authors showed that AcPA activity was highest in lupin grown under P deficiency and that the expression of the gene encoding this enzyme was induced by a decrease in internal P concentrations (Wasaki et al., 2003). Releasing of AcPA is not restricted to lupin roots and this phenomenon was also demonstrated for P-deficient maize in hydroponic culture (Gaume et al., 2001a). In

addition, Asmar et al. (1995) showed higher rhizosphere phosphatase activity which might increase mobilization and depletion of soil organic P in barley. While many authors looked at AcPA, we also investigated the alkaline form (AkPA) of the enzyme and we found that the activity was smaller by a factor of four both in RSC and RSNC, but significantly higher compared to control soil. Acid phosphatases have been reported in plants as well as in microorganisms. In contrast, alkaline phosphatases are produced exclusively by microorganisms and animals (Alef and Nannipieri, 1995). Our results thus suggest that not only the plant produced acid phosphatases, but also the microbial alkaline phosphatases contribute to the improvement of P desorption from the soil matrix.

In addition to phosphatases, release of organic acids from roots is another important mechanism which increases P acquisition by plants (Dinkelaker et al., 1989; Jones, 1998). Citrate and malate have been reported to be secreted from white lupin cluster roots in a growth-stage dependent manner (Massonneau et al., 2001; Neumann et al., 1999 and 2000). For experimental reasons, the different growth stages could not be differentiated in our system, but the presence of both citrate and not only malate indicates that mature cluster roots were also harvested here. In our study, fumarate was more abundant than citrate and malate. Fumarate has previously been observed in the root exudates of lupin: Cawthray (2003) found comparable amounts of fumarate malate and citrate in exudates of soil grown white lupin, while Neumann and Römheld (1999) found traces of fumarate in root exudates of white lupin, as well as in wheat, tomato and chickpea. The possible implication of fumarate in P acquisition is much less documented than it is the case for citrate and malate. Imas et al. (1997) observed that P deficiency induced an exponential increase of fumarate in tomato exudates, which suggests that this organic anion also could be involved in P acquisition. It may complex soil ions and/or could replace P on ligand exchange surfaces as reviewed by Jones (1998) for citrate, malate and oxalate.

The low amounts of organic acids we found in the soil samples, *i.e.* several micrograms compared to several milligrams in the roots themselves (data not shown), could be explained by their rapid sorption on the soil's solid phase which is highly pH dependent as revealed by Jones and Brassington (1998). In our case, carboxylates could be retrieved

in significant amounts only from rhizosphere soil fractions. This is for sure a consequence of the root secretion activity but in addition, the microbial degradation rates of these phosphorus-chelating agents may be highly variable and more important at a larger distance from roots, especially in the case of white lupin, where the pH strongly decreases in the direct proximity of cluster roots. This pH decrease in the rhizosphere of white lupin cluster roots is due to a proton extrusion which occurs concomitantly to the secretion of citrate (Sas et al., 2001). In addition to the role of these protons in maintaining charge balance through compensation of the negative charges of the secreted organic anions, this pH decrease might also have an indirect role in reducing microbial consumption of citrate and malate. We have previously shown that in the rhizosphere of cluster roots, bacterial abundance is significantly reduced during the root stage where most of the citrate is secreted (Weisskopf et al., 2005) and this support the hypothesis that the decrease of pH could be partly responsible for the lower abundance of bacteria at the stage where citrate secretion takes place.

In conclusion, our results showed that the rhizosphere of white lupin, and especially the vicinity of cluster roots was characterized by i) important mineralization processes over the year of incubation ii), high acid phosphatase activity as well as alkaline phosphatase activity, demonstrated to our knowledge for the first time in white lupin rhizosphere and iii) high levels of fumarate, citrate and malate, with citrate only present in the rhizosphere of cluster roots.

We believe that our one year study in microcosms helped to understand biological processes that could occur at a larger scale of time. In addition, our design allowed separation of the actual bulk soil in the side-arms and the rhizosphere soil near the roots. As a matter of fact, our results proved that the soil collected in the central tube (BS) was more or less influenced by the roots, assessing the problem of root proximity in small pots as mentioned by Gregory (2004). The next step of this research is probably the use of mesocosms in the field under annual variations of temperature, natural night/day cycles and rainfall events. In addition, other factors should be taken into account to better understand the P retention as the soil texture, especially the amount of adsorption sites,

e.g. clay particles or organic matter. The implication of the soil fauna and microflora and its interaction with the soil/root system should also be monitored. It is well known that microbes consume root exudates but they are also responsible for the production of organic acids that may help the mobilization of P (Rozycki, 1985 *in* Jones, 1998). Attention must be also devoted to earthworms, as keystone soil organisms involved in i) regulation of nutrient cycling through the dispersal and the stimulation of soil microbial activity associated with passage through the intestinal tract and in ii) the distribution and the mixing of organic matter and soil mineral particles (Edwards and Bohlen, 1996; Lavelle and Spain, 2001). Earthworm surface-casts collected from grassland pasture were found to contain 3 times more water extractable P than the surrounding soil (Sharpley and Syers, 1976 and 1977). In addition, the ingestion and the thorough mixing of soil in the intestinal tract of *Lumbricus rubellus* and *Aporrectodea caliginosa* favor the dissolution of phosphate rock and thus the availability of the derived-P in the soil (Mackay et al., 1982). Finally, in a situation where P fertilizers should become less and less available, complementary agronomic measures in agroecosystems may help to improve P availability. Horst et al. (2001) suggested the integration of P-mobilizing plant species as inter-crops or planted in rotations. *Lupinus albus* appears to be a promising candidate for such an intercropping system, since in addition to its efficient P mobilization, it is also able, as a leguminous plant, to fix nitrogen in symbiotic association with Bradyrhizobiae. In addition, the use of lupin shoots as green fertilizer and the slow mineralization of organic matter from shoot residues left on the soil might promote nutrient recycling and decrease losses by leaching or water runoff.

2.3.3. Synthesis – Discussion - Outlook

In this chapter, we presented a study on phosphorus uptake and dynamics investigated in white lupin cultivated in microcosms for one year. The results obtained underline the fact that cluster roots are the preferential site of phosphate solubilization: we observed significantly lower amounts of extractable P in the rhizosphere soil of cluster roots as compared with the rhizosphere soil of non cluster roots, which suggests that P uptake was more efficient around cluster roots. In addition, phosphatase activities were highest in the rhizosphere soil of cluster roots and so were the amounts of citrate. To see such strong differences between cluster and non cluster roots was all the more interesting when considering that cluster root stages could not be discriminated. Thus, we have to assume that the effect of mature, "active" cluster roots was diluted through the pooling of juvenile, senescent and mature cluster roots, but still was very significant when comparing P uptake, phosphatase activities or citrate secretion in cluster and non cluster roots.

The results obtained were expected and in agreement with previous studies on cluster roots physiology, but to my opinion, the important point here is that they confirm the occurrence also in soil grown plants of the mechanisms related to P solubilization, which had been described and studied mostly in hydroponic systems. In this sense, this long-term experiment with soil-grown plants constituted an important step towards a better understanding of P dynamics in a field context.

The interpretation of the changes observed for the different incubation times was not evident for all parameters. For bioavailable (bicarbonate extractable) phosphate, a transient increase in all samples was observed at half the incubation time (5-6 months cultivation), followed by a constant decrease till the end of the experiment, which could reflect the consequence of P uptake by the successive lupin generations. As for organic phosphate, the same tendency was observed, but interestingly this time only in the rhizosphere of cluster roots. As expected, these organic P levels correlated nicely with the acid phosphatase activities measured in function of time and root proximity. These high

phosphatase activities in the rhizosphere of cluster roots again confirmed previous findings in hydroponic systems (Wasaki et al., 1999, 2003) and showed the ability of white lupin cluster roots to acquire phosphate through hydrolysis of soil organic compounds, in addition to the acquisition of phosphate from the inorganic pool through secretion of organic acids.

The amounts of organic acids quantified in non cluster roots were surprisingly not lower than for cluster roots, except for citrate, which was only detected in cluster root associated soil. These results indicate on the one hand that non cluster roots also secreted substantial amounts of organic acids and on the other hand that due to the "dilution effect" caused by harvesting all cluster root stages together, cluster roots considered as a whole did not account for a larger organic acid secretion that non cluster roots. Apparently, citrate secretion was only happening to detectable levels in cluster roots. The same variability in function of incubation time was observed for organic acids as for phosphate contents and phosphatase activities: a general decrease from the beginning to the middle of the experiment, with an increase towards the end, especially at the 12^{th} month.

The fact that all parameters followed a similar pattern in function of incubation time and especially the fact that, in the case of extractable P amounts, this evolution was also true for the bulk soil samples (side-arms) of lupin and even of control microcosms lead to think that changes might have been caused by a variation in the growth and incubation conditions. It will be very interesting to see if this pattern of temporal changes can also be observed at the level of the microbial communities living in these different soil samples.

2.4. SECRETION OF PHENOLICS

2.4.1. Introduction

2.4.1.1. Interest of studying phenolic compounds in white lupin's cluster roots

In this chapter, we analyzed the phenolic compounds secreted by white lupin's cluster roots (2.4.2.) and investigated the impact of different bacterial and fungal elicitors on the secretion of phenolics by white lupin seedlings (2.4.3.). In contrast to the secretion of organic acids, which has received a lot of attention during the last decades (Dakora and Phillips, 2002; Dinkelaker et al., 1989, 1997; Gerke et al., 1994, 2000; Jones and Darrah, 1994; Ryan et al., 2001), other components of cluster root exudates have been much less studied. Based on previous indications (Neumann et al., 2000) that phenolics might be secreted in higher amounts in cluster roots than in non cluster roots, we decided to analyze the phenolics produced in and secreted from cluster and non cluster roots, with a detailed comparison of the evolution of phenolic secretion and contents during the different stages of cluster root development. We focused on isoflavonoids, since they are the most abundant secondary compounds in leguminous plants. Moreover, isoflavonoids have been reported to be involved both in plant nutrition (Dinkelaker, 1995; Dixon and Paiva, 1995; Marschner, 1995) and plant microbe interactions (Dakora and Phillips, 1996; Paiva, 2000), which are both of interest to us and constitute the two main topics of this thesis.

2.4.1.2. State of research in white lupin's isoflavonoids

Except in white lupin, where phenolics have been reported to be more highly secreted in cluster roots (Neumann et al., 2000) and especially present in high amounts in iron-deficient plants as compared with phosphate-deficient plants (Hägstrom et al., 2001), no information is available about phenolic secretion in other cluster rooted species. In white lupin, isoflavonoids have been previously characterized in detail in the shoots and in the roots (Katagiri et al., 2000; Ingham et al. 1983; Stobiecki et al., 1999; Tahara et al., 1984). However, these studies were never conducted in P deficient conditions and

consequently, cluster roots were not investigated. Moreover, many analyses were carried out after methanol extraction of ground tissues, without taking into account the root secretion of these compounds. More recently, reports on secretion of phenolics by white lupin started to appear in the literature (Jung et al., 2003; Kneer et al., 1999 in yellow lupin; Pislewska et al., 2002), except for studies dealing with elicitation, where secreted phenolics were taken into consideration earlier (see below).

The available data about white lupin's isoflavonoids published so far gave us a useful start for the setup of the HPLC analysis, but we soon realized that the isoflavonoid profiles we obtained from cluster roots were very different from the profiles described in the literature. The putative reasons for this observation are discussed in detail in chapter 2.4.2., but the fact that most likely accounts for this discrepancy is the use of different cultivars of white lupin.

2.4.1.3. Elicitation of isoflavonoid secretion

The production and secretion of secondary compounds involved in plant defense rarely occur on a regular basis, but are more often a response to an environmental challenge through the presence of a pathogen. This phenomenon is called "elicitation" and has been the object of many studies in the last few years. It was also investigated in the case of white lupin, by Wojtaszek and Stobiecki (1997), as well as by Gagnon and Ibrahim (1997), who obtained enhanced isoflavonoid root secretion after elicitation with yeast, chitosan, rhizobiae or copper chloride. We asked the question whether microorganisms isolated from white lupin's rhizosphere, and as such adapted to white lupin cluster roots, would also elicit an enhanced isoflavonoid secretion in white lupin. To assess this question, we investigated the effect of bacterial and fungal strains isolated from white lupin's rhizosphere on the secretion of isoflavonoid by white lupin seedlings. We also tested collection strains of bacterial and fungal potential lupin pathogens, in the idea that they might elicit a stronger response from the plant than the neutral or even plant growth promoting bacteria or the fungi isolated from the rhizosphere soil of healthy white lupin plants.

2.4.2. Phosphate supply, root type and cluster root stage influence isoflavonoid secretion in white lupin

To be submitted to New Phytologist

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2.4.2.1. Abstract

In order to cope with soils where phosphate is sparingly available, white lupins develop special root structures called cluster roots, which enable phosphate solubilization through secretion of organic acids and concomitant rhizosphere acidification. In this study, we used a LC/MS approach to monitor the production and secretion of isoflavonoids in white lupin cluster roots in a hydroponic culture system. The major compounds secreted by cluster roots were identified as the two aglycones genistein and hydroxygenistein and their corresponding mono- and diglucoside conjugates. No prenylated isoflavones were recovered from white lupin cluster roots. We could observe that phosphate deficiency generally increased the amounts of secreted isoflavonoids and that cluster roots secreted more isoflavonoids, and especially more genistein, than non cluster roots. We investigated the pattern of isoflavonoid content and secretion in developing cluster roots and observed quantitative differences in the secretion levels in function of root stage. In contrast to the secretion of citrate, which is most important in mature cluster roots, isoflavonoids were secreted in highest amounts at the juvenile and immature stage, prior to citrate secretion.

2.4.2.2. Introduction

In nature, plants often are growing in soils with sparingly available phosphate. To cope with this problem, they have evolved several tolerance mechanisms (Raghothama, 1999; Schachtman et al., 1998). Two main strategies are the association with mycorrhizal fungi on the one hand and the formation of particular root structures, called cluster roots or proteoid roots (Purnell, 1960) on the other hand. Cluster root formation may not be as common among plants as mycorrhizal symbiosis but it is still a very efficient strategy to cope with phosphate deficiency and does not depend on the fungal counterpart. Cluster roots release high amounts of organic acids into the rhizosphere (Lamont, 2003; Neumann and Martinoia, 2002; Roelofs et al., 2001; Veneklaas et al., 2003) and these organic acids solubilize phosphate mainly by acidification in calcareous soils and by chelation in acidic soils (Dakora and Phillips, 2002; Dinkelaker et al., 1989, 1997; Gerke et al., 1994, 2000; Jones and Darrah, 1994; Ryan et al., 2001). Cluster root physiology and ecology have been intensively studied during the last decade (Keerthisinghe et al., 1998; Lamont, 2003; Neumann and Martinoia 2002; Shane et al., 2003a, 2004; Shane and Lambers, *in press*; Skene, 1998; Watt and Evans, 1999) and a particular interest has been devoted to the secretion of organic acids. However, not much attention has been paid so far to other secreted molecules like phenolics, despite their potential role in plant nutrition (Dinkelaker, 1995; Marschner, 1995) and plant microbe interactions.

Phenolics accumulation is a well-known symptom of nutritional stress and different classes of phenolic compound are produced depending on the element lacking: phosphate deficiency is known to induce anthocyanin accumulation; phenolic acids may be overproduced when iron is lacking and flavonoids as well as isoflavonoids have been linked to nitrogen stress (Dixon and Paiva, 1995). Previous reports have studied the impact of phenolics on heavy metal tolerance, in the case of aluminium in maize (Kidd et al., 2001), or copper in alfalfa (Parry et al., 1994) and white lupin (Jung et al., 2003). There, it was shown that isoflavonoids are able to bind copper ions and it was postulated that this might reduce copper toxicity. This phenomenon might be of general interest for cluster rooted species. Exudation of organic anions is often accompanied by a rhizosphere

acidification. For many heavy metals, solubility increases when pH decreases and phenolics might help reducing this side-effect of phosphate solubilization.

In addition to their role in iron nutrition (Moran et al., 1997; Römheld and Marschner, 1983; Zhang et al., 1991) or heavy metal tolerance (Jung et al., 2003; Schutzendubel et al., 2001), phenolics, and especially flavonoids, also play a major role in plant microbe interactions (Paiva, 2000). In legumes, isoflavonoids are the most abundant class of phenolic compounds and they have been reported to be involved in many plant microbe interactions. On the one hand, they can attract mutualistic microorganisms like nitrogen-fixing bacteria (Dakora et al. 1993) or mycorrhizal fungi (Hirsch and Kapulnik, 1998). On the other hand, many isoflavonoids are involved in the defense response against potential soil-borne bacterial or fungal pathogens. For a review on the various biological roles of isoflavonoids in plant microbe interactions, see Paiva (2000) or Dakora and Phillips (1996). In the context of cluster roots, where the phosphate acquisition relies mostly on the secretion of organic acids, isoflavonoids may play a very important additional role, namely the reduction of microbial growth and thus the protection of the plant's phosphate chelating agents against degradation by rhizosphere bacteria and fungi.

Previous studies on phenolics, and especially isoflavonoids, have been conducted in the cluster-rooted species white lupin. However, these studies either focused on the precise profiling and structure elucidation of the various isoflavonoids in white lupins grown in P sufficient conditions and thus without cluster roots (Bednarek et al. 2001; Jung et al., 2003; Katagiri et al., 2001; Pislewska et al., 2002; Sakasai et al., 2000; Wojtaszek et al., 1993, 1997) or did take into account phenolics only as a general class of compounds in the case where P-deficient white lupins forming cluster roots were studied (Dinkelaker et al., 1995; Neumann et al., 2000). Till now, no study investigated the isoflavonoids produced and secreted by the cluster roots of white lupin plants.

In the present work, we analyzed the isoflavonoid contents and secretion in white lupin's cluster roots and followed the evolution of these contents and secretion as the cluster roots were growing. In white lupin, cluster roots can be separated into four different

growth stages (Neumann et al., 1999): the juvenile, the immature, the mature and the senescent stages. These stages differ in quantity and quality of organic acid secretion: at the juvenile stage, cluster roots are still growing and secrete little amounts of malate. After two to three days, the cluster root comes to the immature stage, where the root full size is reached but no secretion of organic acids occurs. The high secretion of organic acids, mainly citrate, occurs at the mature stage. There, a concomitant release of protons causes rhizosphere acidification. However, acidification and carboxylate secretion are two separate processes. Carboxylates are secreted in the unprotonated form through recently identified channels (Kollmeier et al., 2001; Sasaki et al., 2000; Zhang et al., 2004), whereas acidification is due to the activation of the plasma membrane proton pump (Yan et al., 2002). At the senescent stage, almost no carboxylates are secreted any more.

These precisely defined cluster root growth stages, as well as the fast growth and high root biomass production of white lupin make it a good model to study root-related processes and to follow the spatio-temporal evolution of the secretion physiology. In the present work, we wanted to extend the understanding of cluster root secretion physiology beyond the carboxylates to secondary compounds and we chose to focus on isoflavonoids for their abundance in legumes and their potential role both in nutrition and plant microbe interactions. We used a LC/MS approach to characterize the isoflavonoids present in and secreted from the roots of white lupin. In leguminous plants, isoflavonoid glucosides are partially present as acylated conjugates. Glucosides acylated with aliphatic acids are labile compounds and their acylation/deacylation occurs easily during isolation and purification (Barnes et al., 1994). LC/ESI/MS has been previously used for the detection of isoflavonoid glycosides and free aglycones in extracts of white lupin roots (Bednarek et al., 2001; Stobiecki et al., 1999) as well as in red clover (Lin et al., 2000). Utilization of LC/MS systems overcomes the problems related to the identification of acylated isoflavonoid glycosides. On the basis of m/z values of protonated $[M+H]^+$ and fragment ions, it is possible to identify an acyl substituent linked to a sugar moiety in an isoflavonoid glycoside. On the basis of fragment ion masses created after cleavage of glycosidic bonds, sizes of sugars and aglycone moieties can be estimated.

We have taken advantage of the development of high pressure liquid chromatography (HPLC) coupled to atmospheric pressure ionization (API) with electrospray interface (ESI) mass spectrometry (MS) to facilitate the identification of conjugated isoflavonoids in cluster roots without the necessity to separate all the compounds. We used this analytical tool to address the following biological questions: i) would phosphate nutrition induce changes in the quantity and quality of the isoflavonoid produced and secreted, ii) would the isoflavonoid composition be different between cluster and non cluster roots and iii) would the changing pattern of secretion observed for carboxylates along growing cluster roots also hold true for isoflavonoids.

2.4.2.3. Material and methods

Chemicals

Methanol used for liquid extraction was distilled before use. HPLC grade acetonitrile was purchased from SDS (Peypin, France). *β*-glucosidase and genistein were purchased from Sigma (Germany).

Plant material and harvest of different root parts

White lupin (*Lupinus albus* L. cv. Amiga; Südwestdeutsche Saatzucht, Rastatt, Germany) were grown either in presence (+P) or absence (-P) of P source, as previously described by Massonneau et al. (2001). Plants were grown at 22°C and 65% relative humidity with a light period of 16 h at 200 µmol m^{-2} s^{-1}. For the separation between cluster and non cluster roots (without differentiation of the cluster root stages), cluster roots of all developmental stages were harvested and pooled together, while non cluster roots consisted of entire secondary roots without cluster roots. The different stages of cluster roots were harvested as described by Massonneau et al. (2001). In order to differentiate the developmental stages of root clusters, the root system was immersed in a pH-indicator solution, which indicates acidification in mature cluster roots (Neumann et al., 1999). To

verify if the cutting step in the root harvest procedure would influence the pattern of isoflavonoid secretion, we compared phenolics extracted from cut vs. non cut entire root systems of white lupins. Although the amounts of isoflavonoid recovered were higher in the cut root system, the pattern and proportions of the different secreted compounds were globally the same in both cases (data not shown).

Extraction of phenolic compounds and HPLC analysis

Excised root parts were washed in distilled water and subsequently incubated in four ml water for one hour at room temperature under gentle shaking to allow the secretion of root exudates. The root exudates were collected and frozen at -80 °C. After freeze-drying, 2.5 ml methanol 80 % were added in four steps (1 ml and subsequently three times 0.5 ml), each step was followed by vigorous shaking and filtration at 0.45 µm (Schleicher & Schuell etc). After solvent evaporation, extracts were resuspended in the first HPLC solvent (A) in proportion to the root fresh weight (0.75 µl . mg root fresh weight $^{-1}$ for the secretion samples and 1.5 µl mg root fresh weight $^{-1}$ for the content samples). 50 µl of the samples were then injected into a reversed phase C 18 column (NUCLEOSIL 250 x 4.6 mm, 7.0 µm) for analysis. The elution solvents consisted of water, acetonitrile and acetic acid in the following proportions: 93:5:2 (A) and 23:75:2 (B). Solvent gradient started with 10 % of B and reached 100 % in 25 minutes, with a flow rate of 0.4 ml.min^{-1}. Absorbance was monitored at 263 nm. For quantification of the secreted amounts of genistein, peak 13, peak 16 and malonyl-glucosyl-genestein, calibration curves were elaborated with the isolated pure compounds. All analyses were performed with 3-4 replicates, each replicate representing the harvest of about five boxes containing 12 plants each.

Structure elucidation
LC-ESI-MS analysis

A Hewlett Packard 1100 HPLC system equipped with a binary pump was used with a photodiode array spectrophotometric detector and an autosampler, all controlled by the

Agilent Chemstation software. Separation was performed on a Macherey-Nagel (Düren, Germany) Nucleosil 100-5 C_{18} column (254 x 4 mm i.d; 5 µm) protected by a pre-column of the same material. Gradient elution was performed by varying the proportion of solvent A (MeCN/H_2O/AcAc; 5/93/2; v/v/v) and solvent B (MeCN/H_2O/AcAc; 75/23/2; v/v/v). Starting at 10 % of solvent B, the proportion was programmed to reach 52 % in 16 min, 75 % in 22 min, 100 % in 25 min and solvent B was maintained at 100 % for another 5 min. The total analysis time was 40 min including column wash and stabilization. The flow rate was set to 0.4 $ml.min^{-1}$ and detection to 254 nm, 264 nm and 320 nm.

HPLC-ESI/MS analyses were performed with an Agilent 1100 series LC/MSD Trap instrument equipped with an electrospray ionization source. For positive and negative ionization mode, the pressure of the nebulizer (nitrogen) was set at 50 psi, dry gas flow at 7 ml min^{-1} and the temperature of the drying gas (N_2) at 300 °C. The capillary voltage was at 3660 volts in positive and 2900 volts in negative ionization mode. The voltage of the skimmer lens and the entrance lens in the ion source were automatically optimized by direct inlet of a solution of genistein, hydroxygenistein and genistein glucoside isolated from white lupin leaf extracts. For HPLC analysis, genistein, hydroxygenistein, and genistein 7-O-glucoside were also isolated from leaf extracts. Identification of these compounds, used as standards, was based on NMR and MS spectral data (Ingham, 1976; Ingham et al., 1983; Murthy et al., 1986 and Tahara et al., 1984).

Hydrolysis

2 mg of methanolic extract from the roots of white lupin were added to 6 mg of *β*-glucosidase in 4 ml of acetate buffer pH 5 at 37 °C for 12 h. The sample was then extracted with ethyl acetate, dried and dissolved in 200 µl of solvent A. 10 µl were injected in HPLC for analysis.

Statistical analyses

Analyses of variance were performed with the S-Plus 6 Statistical Software (Insightful Corporation, Seattle, WA, USA) with an interval of confidence of 95 %. We used one-way ANOVA to test the general influence of P nutrition or cluster root stage for statistical relevance and the Student's T test for pair-wise comparisons.

2.4.2.4. Results

Profiling of isoflavonoids conjugates in roots and root exudates

The enzymatic hydrolysis of a root exudate extract with *β*-glucosidase yielded the free aglycones genistein and hydroxygenistein, establishing the anomeric configurations of the glycosidic linkages between aglycones and sugar moieties as β and confirming the absence of prenylated isoflavonoids in the extract (data not shown). In the present study, the HPLC analytical conditions were determined after investigating the effects of various combinations of chromatographic solvents, flow rates and gradient elution conditions on the separation of isoflavonoids from root exudate extracts. The best analysis conditions were obtained with an acetonitrile gradient in water using acetic acid as modifier. Acidification of the mobile phase not only improved resolution for liquid chromatography but also played an important role in the ionization process by decreasing the relative contribution of the $[M+Na]^+$ ions in the MS, as previously observed by Stobiecki et al. (1999).

For MS condition optimization, direct injection electrospray mass spectra of a mixture of the standard isoflavonoids at a concentration of 1 $\mu g.ml^{-1}$ each were measured in MeCN:H2O:AcAc (1:1:2 %) in positive and negative ionization mode. The relative intensities of the molecular species and fragment ions were dependent on capillary voltage, on the temperature of ES source and on the drying gas. Ion chromatograms resulting from LC-ESI-MS analyses of the root exudates extract are shown in Figure 28, together with LC-UV traces obtained in parallel.

The isoflavonoid conjugates were detected on the basis of their UV absorption at λ 263 nm and 350 nm (Figure 28). Twelve peaks of isoflavonoid glycosides, identified earlier in white lupin or other lupin species, were recognized (Franski et al., 1999; Shibuya et al., 1991) based on UV spectra, *m/z* values of protonated molecules $[M+H]^+$ and fragment ions Y_0^- created after cleavage of the glucosidic bonds, between sugar and aglycone (Figure 29).

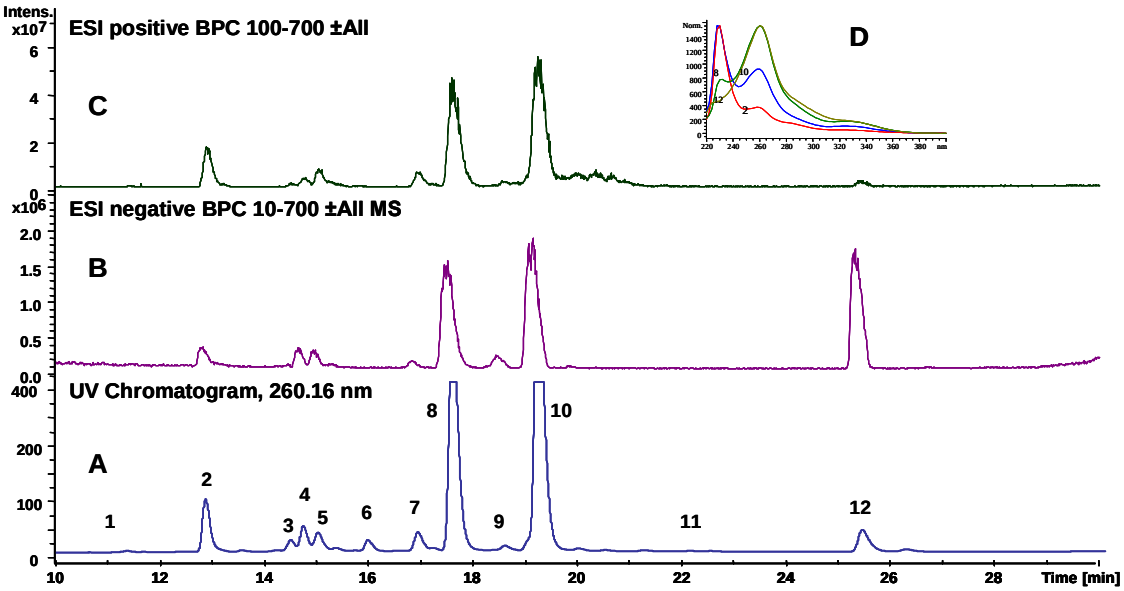


Figure 28: LC-ESI-MS analysis of white lupin root exudates
LC-UV chromatogram (A), LC-ESI-MS [M-H]- ion (B) and LC-ESI-MS [M+H]+ ion (C) base peak chromatograms of root exudates extract. UV spectrum of major compounds (D).

During analysis of the compounds in the positive ion mode, isoflavonoid conjugates showed $[M+H]^+$ as the main peak and no decomposition was observed. The optimal conditions to produce the ion $[M+H]^+$ were found at 3660 volts, 300°C and 7 $l.min^{-1}$ for the capillary, drying and nebulizer gas (Figure 30 C). In contrast, in the negative ion mass spectra, Yn type fragment ions were mainly observed corresponding to the genistein moiety Y_0^- m/z 269 or the hydroxygenistein moiety Y_0^- m/z 285 at 2900 volts. The consecutive loss of 162 amu from the deprotonated $(M-H)^-$ species corresponded to the cleavage of a glucose (Glc) moiety, whilst the elimination of 204 amu was connected

with the elimination of an acetyl-glucoside (Ac-Glc) and 248 amu with the elimination of the malonyl glucosyl (Mal-Glc) moiety. The abundance of the fragments $[M-H-Glc]^-$ and $[M-H-Mal-Glc]^-$ for O-Glc and O-Glc-Mal was very important compared to $[M-H]^-$, giving rise to an ion corresponding to the aglycone. (Figure 30 B)

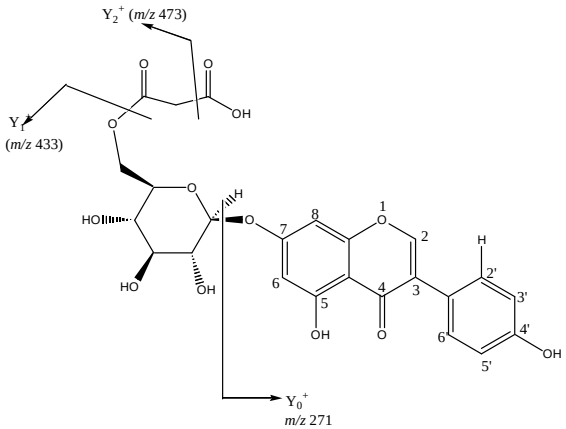


Figure 29: Nomenclature and diagnostic fragmentation of O-Mal-Glc-genistein

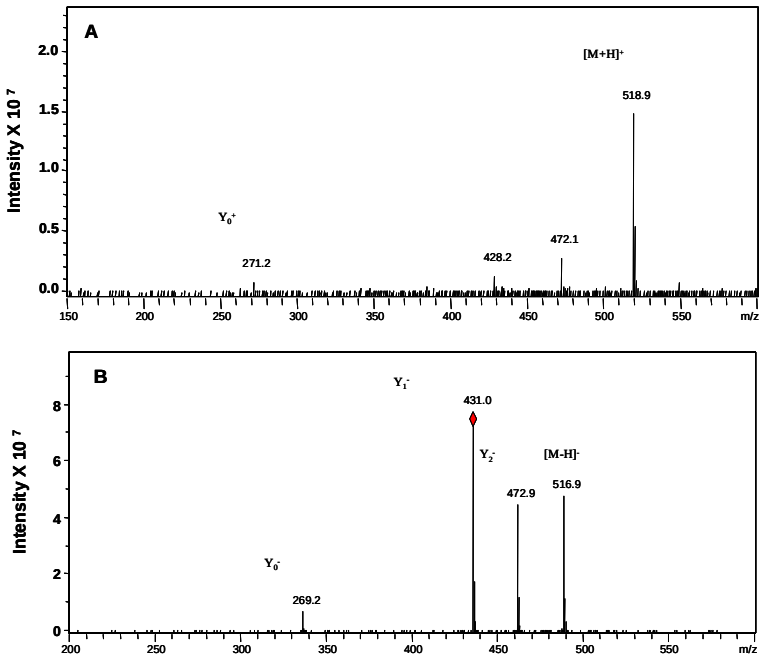


Figure 30: ESI mass spectra for O-Glc-Mal-genistein

(A)- positive ionisation mode [M+H] ion at m/z 519, (B) negative ionisation mode Y0-fragment ion at m/z 269 after cleavages of glycosidic bonds.

Table I: Isoflavonoids and their glucosides identified in lupin root exudates

Peak numbers	Compounds	$[M+H]^+$
1	hydroxygenistein 7-*O*-diglucoside	611
2	genistein 7-*O*-diglucoside	595
3	hydroxygenistein 6"-*O*-malonyl-glucoside	697
4	genistein 6"-*O*-malonyl-diglucoside	681
5	genistein 6"-*O*-malonyl-glucoside 4-*O*-glucoside	681
6	hydroxygenistein 7-O-glucoside	449
7	hydroxygenistein 6"-*O*-malonyl-glycoside	535
8	genistein 7-*O*-glucoside	433
9	genistein 4-*O*-glucoside	433
10	genistein 6"-*O*-malonyl-O-glucoside	519
11	hydroxygenistein	287
12	genistein	271

The abundance of the fragment ions Y_0^- at *m/z* 269 (genistein) and 285 (hydroxygenistein) increased at higher capillary values (data not shown). These ions were of importance because they allowed determination of the mass of the aglycone. It was not possible to establish unambiguously the positions of the sugar substitution on genistein diglucoside, and two possibilities are suggested: the compound could be genistein 7-*O*-diglucoside, or it may occur as a structure deduced from literature data which indicates that glycosylation might occur on the 4' and 7 hydroxyl group of genistein, such a structure having been identified in hairy root cultures of lupins (Berlin et al., 1991). The structure of the major compound 6"-*O*-Glc-Mal was supported by the presence of two fragments ions in the MS besides the $(M-H)^-$ ion at m/z 517 (genistein) and 533 (hydroxygenistein). These ions resulted from the consecutive elimination of an CHOCHCOOH fragment (86 u) deriving from the malonate group, giving rise to an ion at m/z 431 and 447, and cleavage of the glucosidic bond with change retention on the aglycone moiety leading to an ion at m/z 269 and 285. The malonyl group is most

probably in 6”-position of their sugar part and this hypothesis is supported by the fact that so far only 6”-malonates of the isoflavonoid glucosides were reported from *Lupinus albus*. The absence of prenylated isoflavonoids was confirmed by the absence of elimination of isobutene (56 u) as a result of the presence of the isopentenyl substituent producing ions of the $[MH\text{-}iC4H6]^-$ type compounds. Beside the free aglycones genistein (12) and hydroxygenistein (11), the root exudate extracts contained the mono (6, 8, 9) and diglucosyl (1, 2) conjugates and the mono (7, 10) and diglucosyl malonyl (3, 4, 5) conjugates, with the genistein conjugates being the most abundant (Table I).

Isoflavonoid contents and secretion is influenced by P supply

To assess the influence of phosphate nutrition on the contents and secretion of isoflavonoids, phenolics were extracted from white lupin plants grown either in P-deficient of P-sufficient conditions. As previously observed (Shen et al., 2003), few cluster roots also formed in the P-sufficient lupin plants, but much less than in the P-deficient plants. Roots were separated into 4 different fractions, apex of cluster roots, cluster roots, apex of non cluster roots and non cluster roots. Isoflavonoid contents and secretion were analyzed in the four root fractions. Figure 31 shows the total amounts of isoflavonoids, calculated as the sum of the major peaks in the HPLC profiles. When grown under P deficiency, plants produced significantly more isoflavonoids (ANOVA, $P < 0.05$) than when supplied with phosphate (Figure 31 A). This effect was especially marked for non cluster roots and their apex (Student’s t test, $P < 0.05$). In cluster roots and the corresponding apices, no significant difference in isoflavonoid contents could be observed between plants grown with and without phosphate. As observed for the secretion of isoflavonoids, the internal contents were also higher in plants grown in the absence of phosphate (Figure 31 B), except for non cluster roots, where no significant differences were observed. Phosphate nutrition did not affect all isoflavonoids in the same way: in cluster roots for instance, a significant increase in genistein (12) contents (Student’s t test, $P < 0.05$) was observed for P-deficient plants. These plants contained 2.3 mg genistein. g root fresh weight $^{-1}$, while only 0.9 mg. g root fresh weight $^{-1}$ were detected for plants grown in sufficient P conditions. In contrast, no change upon P

treatment could be observed for genistein 6"-*O*-malonyl-O-glucoside (10), which was present at the concentration of approximately 0.2 $mg.g^{-1}$ in cluster roots of both P-deficient and P-sufficient plants. Overall, the ratio of internal contents *vs.* secretion was not altered by the P treatment.

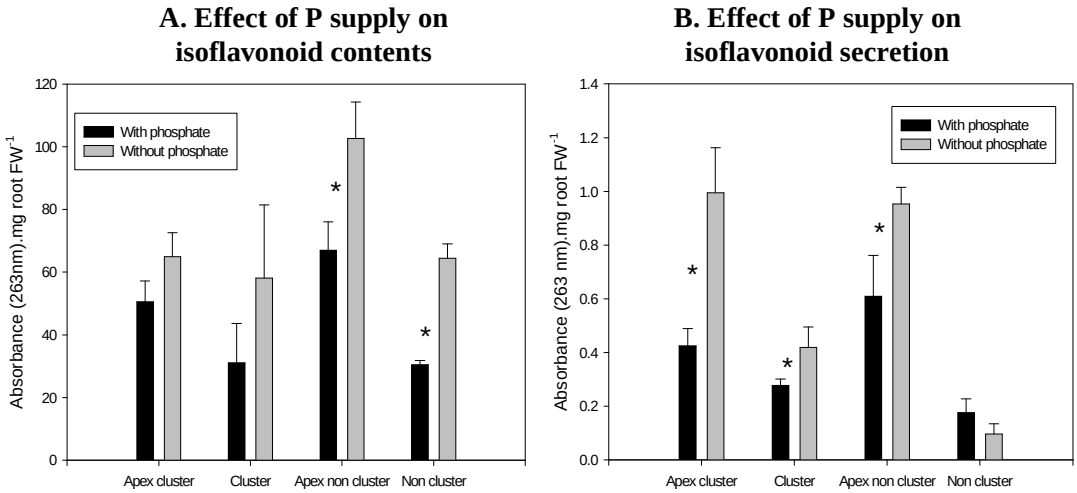


Figure 31: Isoflavonoid contents and secretion in function of P supply

*Isoflavonoid contents (A) and secretion (B) mg-1 root fresh weight in function of P supply. White lupins were grown in hydroponic cultures for 5 weeks under P-sufficient or P-deficient conditions. Roots were separated into cluster roots (apex and roots) and non cluster roots (apex and roots). Root tissues (contents, A) and exudates (secretion, B) were extracted with 80% methanol. Samples were separated on a C18 HPLC column and absorbance was monitored at 263 nm. Total isoflavonoids contents and secretion were calculated as the sum of the areas of all major isoflavonoid peaks in the HPLC profile. Bars represent means of three replicates, * indicate significant differences (Student's t test, $P < 0.05$).*

Isoflavonoid contents and secretion is influenced by root type

The comparison between contents and secretion of four major isoflavonoids in cluster roots and non cluster roots of P-deficient lupin plants is shown in Figure 32. In the internal isoflavonoid pool (Figure 32 A and B), higher levels of genistein (12) were found in cluster roots ($P < 0.05$) than in non cluster roots, but no significant difference could be observed for the other compounds. In contrast, the secretion levels of isoflavonoids were generally higher for cluster roots than for non cluster roots (Figure 32 C and 32 D).

Genistein (12), genistein 6"-*O*-malonyl-O-glucoside (10) and genistein 7-*O*-diglucoside (2) exhibited an enhanced secretion ($P < 0.05$) in cluster roots, as compared to non cluster roots. A similar observation between cluster and non cluster roots could also be made for plants grown in P-sufficient conditions (data not shown).

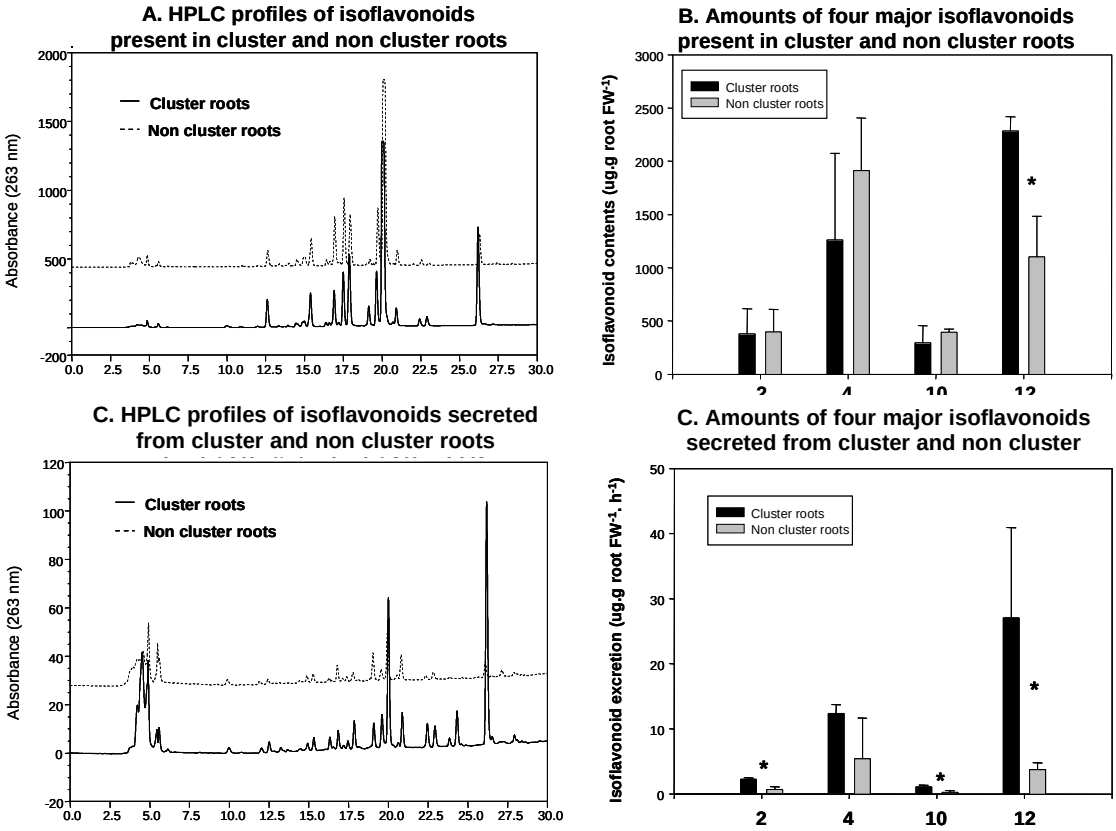


Figure 32: Isoflavonoid contents and secretion in function of root type

*Contents (A, B) and secretion (C, D) of isoflavonoids in function of root type. White lupins were grown in hydroponic cultures for 5 weeks under P-deficient conditions. Roots were separated into cluster roots and non cluster roots. Root tissues (contents, A and B) and exudates (secretion, C and D) were extracted with 80% methanol. Samples were separated on a C18 HPLC column and absorbance was monitored at 263 nm. A and C show the HPLC profiles of cluster roots (bold line) and non cluster roots (dashed line), for contents (A) and secretion (C). For internal contents (A), 20 µl were injected, corresponding to a root weight of 10 mg. For secretion (C), 50 µl were injected, corresponding to a root weight of 50 mg. B and D: Quantification of four major isoflavonoids in µg. g^{-1} root fresh weight for internal contents (B) and in µg. g^{-1} root fresh weight . h^{-1} for secretion (D). 2: genistein 7-O-diglucoside; 4: genistein 6"-O-malonyl-diglucoside; 10: genistein 6"-O-malonyl-O-glucoside and 12: genistein. Bars represent means of three replicates, * indicate significant differences (Student's t test, $P < 0.05$).*

Isoflavonoid contents and secretion is influenced by cluster root stage

In order to investigate whether the developmental stages of cluster roots played a role in isoflavonoid storage and secretion, cluster roots were separated into four fractions: juvenile, immature, mature and senescent cluster roots. In all fractions, isoflavonoid contents and secretion were determined.

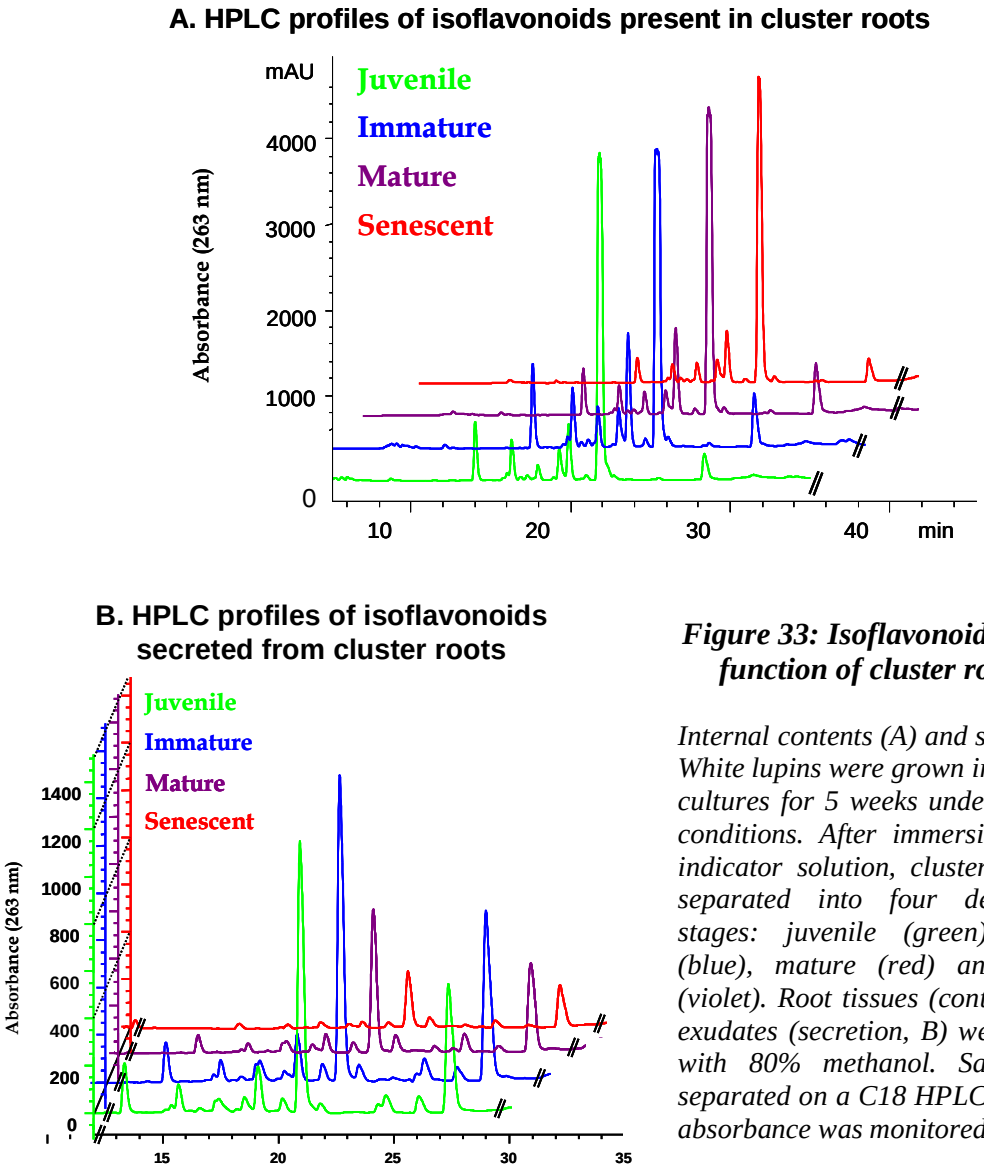


Figure 33: Isoflavonoid profiles in function of cluster root stage

Internal contents (A) and secretion (B). White lupins were grown in hydroponic cultures for 5 weeks under P-deficient conditions. After immersion in a pH indicator solution, cluster roots were separated into four developmental stages: juvenile (green), immature (blue), mature (red) and senescent (violet). Root tissues (contents, A) and exudates (secretion, B) were extracted with 80% methanol. Samples were separated on a C18 HPLC column and absorbance was monitored at 263 nm.

Figure 33 shows the comparison of HPLC profiles of isoflavonoid contents (A) and secretion (B) for all growth stages. The profiles shown here are representative examples of the four replicates we analyzed for each stage. Almost no quantitative changes could be observed for the internal isoflavonoids (Figure 33 A). In contrast, differences were observed in isoflavonoid secretion (Figure 33 B) along the growing cluster roots. In contrast to the pattern of organic acid secretion, which was highest in mature cluster roots, most of the isoflavonoid secretion occurred at the beginning of cluster root development, especially in juvenile and immature cluster roots (Figure 33 B). At the mature stage, the secretion decreased and slowed further down at the senescent stage.

In order to obtain a more quantitative and representative idea of the levels of isoflavonoid contents and secretion, we determined the amounts of the 12 major compounds present in and secreted from the four stages of cluster roots.

Figure 34:

Internal contents and secretion of major isoflavonoids in function of cluster root stage

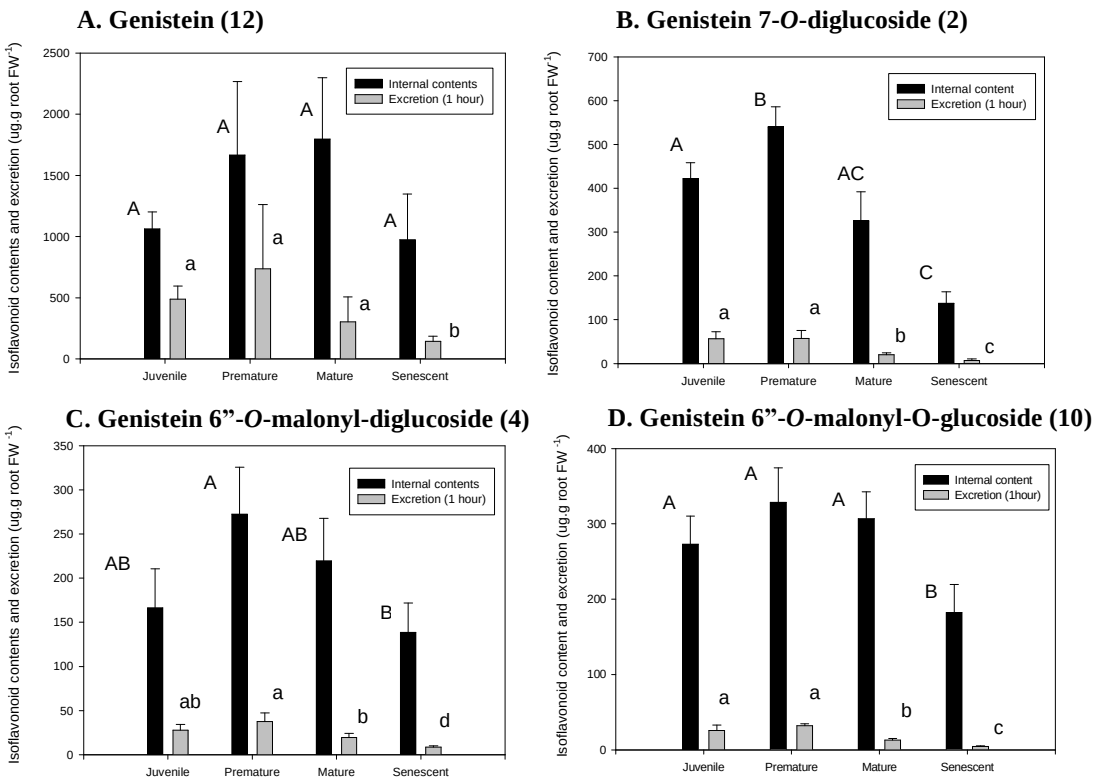


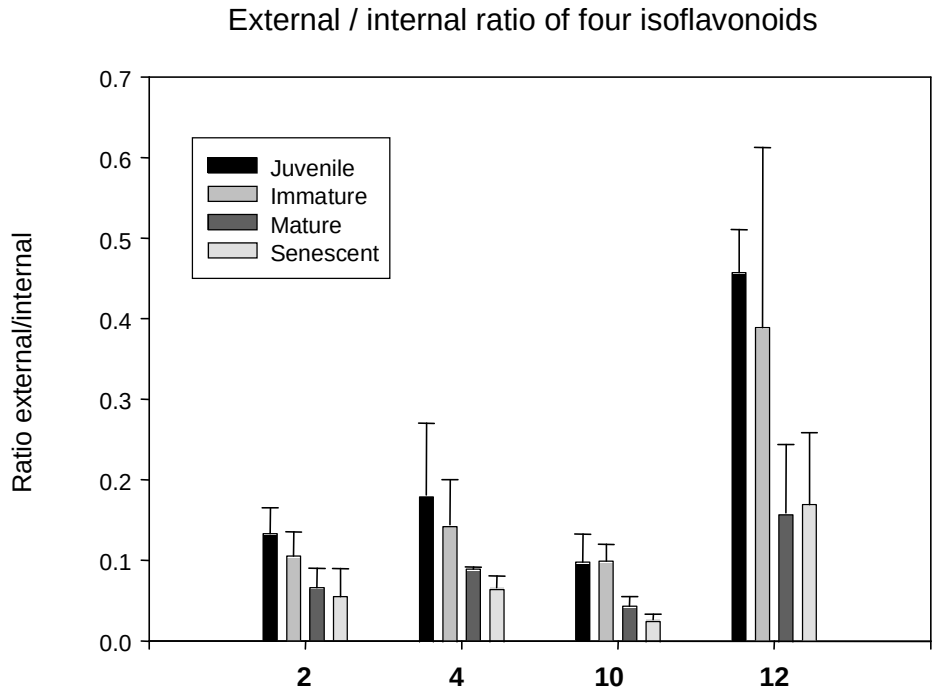
Figure 34: *Quantification of four major isoflavonoids in roots and exudates of white lupin in function of cluster root stage. White lupins were grown in hydroponic cultures for 5 weeks under P-deficient conditions. After immersion in a pH indicator solution (see Material and Methods for more details), cluster roots were separated into four developmental stages: juvenile, immature (premature), mature and senescent. Root tissues and exudates were extracted with 80% methanol. Samples were separated on a C18 HPLC column and absorbance was monitored at 263 nm. Black bars show the internal contents (in µg.g-1 root fresh weight) and grey bars show the secretion (in µg.g-1 root fresh weight .h-1) for genistein 7-O-diglucoside (A), genistein 6''-O-malonyl-diglucoside (B), genistein 6''-O-malonyl-O-glucoside (C) and genistein (D). Bars represent means of three replicates. Different letters stand for statistically different values (Student's t test, P < 0.05).*

Overall, isoflavonoid contents and secretion varied significantly with cluster root stage (ANOVA, $P < 0.05$). Among the 12 major compounds tested for significant changes in function of cluster root stage, 6 showed an altered pattern in contents and 6 in secretion levels. Figure 34 shows the pattern for four of them. Genistein (A) did not show any significant change in the pattern of secretion or in the internal contents. However, compared to the other three, this isoflavonoid displayed the highest secretion vs. internal contents ratio. Internal contents and secretion of genistein 7-*O*-diglucoside showed the same pattern (B): starting high at the juvenile and immature stages, and then decreasing significantly at the mature stage and further diminishing at the senescent stage. While no significant difference could be observed for genistein 6''-*O*-malonyl-diglucoside in internal contents (C), the secretion was higher for juvenile and immature ($P < 0.05$) than for mature and senescent growth stages. In contrast to genistein 7-*O*-diglucoside and to genistein 6''-*O*-malonyl-diglucoside, genistein 6''-*O*-malonyl-O-glucoside (D) showed no difference in contents between the immature and the mature stages, whereas there was a significant decrease in secretion between these two stages.

As already indicated in the HPLC profiles of isoflavonoids secreted from the different cluster root growth stages (Figure 33 B), the secretion mainly occurred at the juvenile and immature stages, and then a decrease was observed at the mature and senescent stages. We calculated the ratios of secretion *vs.* internal contents for four major compounds in order to see whether or not this decrease in isoflavonoid secretion was a consequence of a decreased production. Figure 35 shows the changes occurring in the ratios of secretion vs.

contents as the cluster root is growing. In general, ratios varied significantly with cluster root stage and genistein was more highly secreted than the three other compounds (ANOVA, $P < 0.05$). For all peaks, the ratio was higher (more secretion) in juvenile and immature cluster roots and lower at the mature and senescent stages, suggesting that the decrease in secretion cannot be explained solely by the decrease in synthesis. This decrease at the transition from the immature to the mature stage was significant (Student's t test, $P < 0.05$) for genistein 6"-*O*-malonyl-O-glucoside (10). No changes were observed between the mature and the senescent stages.

Figure 35: External* vs. *internal contents ratio of four isoflavonoids



Ratio of secretion vs. *internal contents of four major isoflavonoids in function of cluster root stage. White lupins were grown in hydroponic cultures for 5 weeks under P-deficient conditions. After immersion in a pH indicator solution (see Material and Methods for more details), cluster roots were separated into four developmental stages: juvenile (black), immature (dark grey), mature (light grey) and senescent (white). Root tissues and exudates were extracted with 80% methanol. Samples were separated on a C18 HPLC column and absorbance was monitored at 263 nm. 2: genistein 7-O-diglucoside; 4: genistein 6"-O-malonyl-diglucoside; 10: genistein 6"-O-malonyl-O-glucoside and 12: genistein. Bars represent means of three replicates.*

2.4.2.5. Discussion

In order to obtain new insights into the secretion physiology of cluster roots, this efficient strategy evolved by some plants to cope with phosphate deficiency, we applied an LC-MS approach to characterize the pattern of isoflavonoids produced and secreted from different stages of white lupin's cluster roots. We investigated the effects of phosphate nutrition, root type and cluster root stage on the quantity and quality of isoflavonoid contents and secretion.

Major isoflavonoid found in white lupin's cluster roots

LC-UV-MS techniques were used for structural elucidation and profiling of flavonoid glycosides in root exudates from *Lupinus albus*. Four diglucosides, six mono-glucosides and two aglycones were identified in the extracts (Figure 28, Table 1). All recognized compounds were reported earlier in white lupin or other lupin species (Shibuya et al., 1991). Hydrolysis of root extracts with β- glucosidase confirmed the presence of only two aglycones, genistein and hydroxygenistein and the absence of prenylated compounds. This absence of prenylated compounds was surprising for us, because earlier reports suggested that prenylated isoflavonoids were present in white lupin roots: Tahara et al. (1984, 1989) isolated two prenylated isoflavonoids (the monoprenylated lupinalbigenin and the diprenylated 2'-hydroxyisolupinalbigenin) from white lupin roots and Bednarek et al. (2001) reported the presence of two monoprenylated isoflavonoids, wighteone and luteone. Since the extraction method and the profiling analysis were comparable between the cited studies and the present work, a possible reason for the differences in the pattern of isoflavonoid recovered might be the fact that different lupin cultivars were used (*Lupinus albus* cv. Bac. by Bednarek et al., *L. albus* cv. Kievskij Mutant by Tahara et al. and Katagiri et al., whereas *L. albus* cv. Amiga was used in the present study). In this study, two groups of isoflavonoid conjugates could be detected on the basis of the m/z Y_0^- ions and the major compounds were related to the genistein, while the hydoxygenistein conjugates were less abundant.

Effect of P supply

Since a lack of phosphate is inducing the formation of cluster roots in white lupin, we wanted to assess if the pattern of isoflavonoids would differ in plants grown in P-deficient *vs.* P-sufficient conditions. We could observe that phosphate deficiency caused a general increase in contents and secretion of isoflavonoids (Figure 31). Even if anthocyanin accumulation is a well-known symptom of phosphate deficiency, the effect of phosphate nutrition on the production and secretion of isoflavonoids at the root level has not been often investigated. To our knowledge, only two studies reported an enhanced phenolic production in plants submitted to phosphate deficiency: Murali et al. in 1983 for soybean plants and Juszczuk et al. in 2004 for bean. Juszczuk and co-workers (2004) found that in bean, phosphate deficiency increased the activity of L phenylalanine ammonia lyase, as well as root exudation of phenolics. However, the chemical nature of the secreted phenolic compounds was not investigated. In the case of nitrogen deficiency, the role of isoflavonoids in the signaling leading to the symbiosis with nitrogen-fixing bacteria is well documented and one cannot exclude that similar compounds might be involved in other kinds of nutrient-deficiency signaling pathways. Supporting this hypothesis, Akijama et al. (2002) found that in melon roots, P-deficiency induced the secretion of a glycosylflavonoid which was involved in the regulation of the association with arbuscular mycorrhizal fungi. This may indicate that flavonoids, and isoflavonoids potentially also, might be involved in P-deficiency signaling as well as in N-deficiency signaling.

Differences between cluster and non cluster roots

By comparing the HPLC profiles of cluster *vs.* non cluster roots grown in P deficient conditions (Figure 32 A and C), it could be observed that the profiles are very similar and only differ in the amounts of some isoflavonoids, like genistein for instance, which is higher in cluster roots. Quantitative differences between cluster and non cluster roots were more pronounced in the secretion than in internal contents, and this suggests that both types of roots are producing the same pattern of phenolic compounds but that cluster

roots are secreting higher amounts. This result confirms the observation of Neumann et al. (2000) that more phenolic compounds were secreted from cluster roots than from non cluster roots and it gives additional information concerning the chemical nature of these phenolic compounds. It seems thus that isoflavonoids, as well as organic acids, are secreted at higher rates by cluster roots than by non cluster roots. The comparison between the two types of roots might have given quite a different picture if plants had been grown in N-deficient conditions. Since in soil-grown plants, nodules mostly occur in root regions, where no clusters are formed (unpublished results), we might have seen differences in the patterns of isoflavonoids in non cluster and potentially nodulating roots, as compared with the cluster roots.

Pattern of isoflavonoid secretion along growing cluster roots

Thanks to the good knowledge of the organic acid secretion physiology of cluster roots, we were able to separate growing cluster roots into well-defined developmental stages (Massonneau et al., 2001). It proved useful to analyze the secreted fractions and not only the root contents, where no significant difference between stages was found except for the senescent stage, which was characterized by a decreased level of isoflavonoids. While most of the organic acid secretion is occurring at the mature stage, we could show in this work that the burst of isoflavonoid secretion is happening before this stage, starting at the juvenile stage and staying high till the immature stage. The fact that this isoflavonoid burst occurs immediately before the secretion of citrate takes on an interesting meaning, when considering the potential anti-microbial role of isoflavonoids (Dakora and Phillips 1996; Stafford, 1997): it could be interpreted as a way of the plant to locally and temporarily reduce microbial density in the rhizosphere of cluster roots, just before the secretion of citrate, and thus to decrease the microbial degradation of this phosphate chelating agent.

Interestingly, this pattern of isoflavonoid secretion along growing cluster roots, starting high at the juvenile and immature stages and decreasing at the mature stage, can be related to the expression and activity of a particular enzyme, the ATP-citrate-lyase

(ACL). As previously reported (Langlade et al., 2002), ACL activity is highest at the beginning of cluster root development and decreases at the mature stage. ACL catalyzes the ATP-dependent breakdown of citrate into oxaloacetate and acetyl-CoA. As suggested by Langlade and co-workers (2002), this enzyme could be responsible for the switch in organic acid preferentially secreted from juvenile and mature cluster roots, by reducing the citrate concentrations and providing the malate precursor oxaloacetate. Moreover, ACL activity also produces acetyl-CoA, in addition to oxaloacetate and this acetyl-CoA might well be used in the biosynthetic pathway of flavonoids. Thus, the down-regulation of a single enzyme could explain two different important changes in the secretion physiology of growing cluster roots: both the switch from malate to citrate at the mature stage and the decrease in isoflavonoids from the immature to the mature stage.

By comparing the ratios of secretion *vs.* internal contents (Figure 35), one can observe that genistein is secreted at a higher ratio than the other compounds. Genistein is the less hydrophilic molecule among our identified isoflavonoids and this may account for the easy diffusion across the plasmamembrane and for the higher secretion. In all four compounds analyzed, there was a tendency for the external *vs.* internal ratio to be higher for the juvenile and immature stages, compared with the mature and senescent stages. This suggests that secretion is not only the consequence of an enhanced production but that an active secretion mechanism is taking place. The isoflavonoid internal contents of juvenile, immature and mature cluster roots were not different (Figure 34). However, at the mature stage, secretion was significantly reduced. This hints at a possible storage mechanism of these non-secreted compounds in the root cells, probably in the vacuole. We are currently investigating the possible involvement of a MATE transporter, which is highly expressed in mature cluster roots, in isoflavonoid transport.

2.4.3. Phenolic secretion in white lupin seedlings is altered after fungal and bacterial elicitation

2.4.3.1. Abstract

In this chapter, we addressed the question whether bacterial and fungal elicitation would alter the profiles of isoflavonoids secreted from white lupin seedlings. We analyzed the effect of bacterial and fungal strains isolated from the rhizosphere of white lupin, as well as the effect of collection strains. We tested the impact of "neutral", PGPR (plant growth promoting rhizobacteria) and potentially deleterious strains of bacteria. While inoculating seeds of white lupin with a suspension of "neutral" or PGPR bacteria caused a decrease in the recovered phenolic compounds exudated, inoculation with either an autoclaved suspension of *E. coli* or a suspension of living and potentially plant pathogenic *Pseudomonas syringae* induced a higher secretion of isoflavonoids. To test the effect of fungal elicitors on the secretion of phenolics by white lupin seedlings, we used fungal strains isolated from the root fraction of juvenile and senescent cluster roots, as well as from the rhizosphere soil fraction of mature cluster roots. While the fungi isolated from juvenile and senescent cluster roots did not alter the profile of secreted phenolics, the mature cluster root strain caused a drastic increase in the isoflavonoid secreted, suggesting a strong plant defense reaction. The impact of potentially pathogenic fungi was also assessed with collection strains: *Fusarium solani* and *Fusarium monoliforme* caused similar changes, with decreased levels of the more hydrophilic isoflavonoids and an increase in the less hydrophilic ones, especially genistein. *Rhizoctonia solani* did not induce any change, in contrast to *Pythium ultimum*, which induced an increased secretion of many isoflavonoids. This study showed that the secretion of phenolics by white lupin seedlings was affected differentially by bacterial and fungal strains. It gave very interesting but preliminary results, which was mostly due to the lack of replicates and the obtained results should be confirmed by further investigations.

2.4.3.2. Introduction

In the previous chapter (2.2.2.), we described the pattern of isoflavonoids produced and secreted by different stages of white lupin's cluster roots and we postulated a possible impact of these compounds in plant microbe interactions. Here, we wanted to investigate the other side of the coin, namely to assess if the presence of microorganisms at the time of seed germination would alter the secretion of phenolics by the seedlings.

Isoflavonoids are known to be involved in plant defense against a wide range of organisms, like herbivores or pathogenic fungi and bacteria (Dakora and Phillips, 1996; Dixon and Paiva, 1995; Paiva, 2000; Smith and Banks, 1986). In the early stage of pathogen recognition, plants respond to the presence of microbes by increasing the secretion of defense-related compounds. This phenomenon, called elicitation, has been the subject of many studies in the last few years (Boudart, 1998; Little and Magill, 2003; Thaler et al., 2002). The defense compounds, which are formed in response to attacks by pathogens, are generally referred to as "phytoalexins" and the causing agents, which may be organisms but also only microbial components acting as signals, like flagellin for bacteria (Meindl et al, 2000; Shimizu et al., 2002) or chitosan for fungi (Akiyama et al., 1994; Grosskopf et al., 1991), are called elicitors.

The elicitation of flavonoid secretion has often been demonstrated in different legumes and in response to different elicitors: reduced glutathione elicited isoflavonoid release from chickpea seedlings (Armero et al., 2001), inoculation with *Fusarium solani* caused an isoflavonoid accumulation in soybean hairy roots (Lozovaya et al., 2004), inoculation of *Colletotrichum trifolii* induced phytoalexin accumulation and increased beta-1,3-glucanase activity in alfalfa (Salles et al., 2002) and inoculation of both pea (Singh et al., 2002) and chickpea (Singh et al., 2003) with two *Pseudomonas* PGPR bacteria increased the synthesis of phenolics and conferred a better protection against pathogenic fungi. In white lupin, elicitor-induced accumulation of isoflavonoids has also been reported: Wojatsek and Stobiecki (1997), as well as Gagnon and Ibrahim in the same year, showed that yeast extract, chitosan, rhizobium suspension and $CuCl_2$ induced higher isoflavonoid

synthesis and secretion. Bednarek et al. (2001) elicited three weeks-old white lupin plants with yeast extract and investigated the changes in the isoflavonoid profiles in the leaves. He found that prenylated isoflavone aglycones were present in higher amounts after elicitation. Prenylated isoflavonoids are known to exhibit high antimicrobial activities, which are probably due to their deep penetration into lipid bilayers (Hendrich et al., 2002). In white lupin, a membrane-associated isoflavone prenyl-transferase has been shown to be involved in the prenylation of isoflavonoids (Laflamme et al., 1993). The enhanced secretion of aglycones upon elicitation has been correlated with the activity of a cell-wall associated beta-glucosidase in white lupins (Pislewska et al., 2002). Isoflavonoids are thought to be stored preferentially in the glycosylated form and to be hydrolyzed into aglycones prior to secretion. In the study of Pislewska et al. (2002), beta-glucosidase activity was induced by yeast elicitor treatment and it was accompanied by changes in the isoflavonoid secretion and accumulation patterns.

In response to this enhanced secretion of phenolic compounds, microorganisms have evolved ways to counteract the plant defense mechanisms and one of these ways obviously is the breakdown of secreted defense compounds. This has been shown for *Eubacterium ramulus* in the context of the human nutrition (Schoefer et al., 2002), but also in the case of rhizosphere bacteria (Barz, 1970; Cooper and Rao, 1995) and fungi like *Aspergillus flavus*, *Botrytis cinerea* and *Ascochyta rabiei* (Tahara et al., 1997). It remains unclear whether these microorganisms only degrade isoflavonoids to detoxify them or if they can use these compounds as a carbon source. To our knowledge, this has been investigated up to now only for simple phenolics secreted from lichens (Stark and Hyvärinen, 2003) and in that case, it could be shown that the soil microbial community indeed used these phenolic acids as carbon source.

The present study was conducted to see if bacteria and fungi isolated from white lupin's rhizosphere would have an impact on the isoflavonoid secretion of white lupin seedlings. We compared the effect of a "neutral" bacterium, isolated from the bulk soil and which did not show any PGPR properties and the effect of a "PGPR" bacterium, isolated from white lupin's juvenile cluster roots and able to produce auxin, as well as to solubilize

phosphate and iron. In addition to the isolated strains, we used two bacterial collection strains: *Escherichia coli* as a "neutral" strain and *Pseudomonas syringae* as a potential deleterious one, which is inhibited by white lupin alkaloids (delaVega et al., 1996). For fungal elicitation, we used three strains isolated from the rhizosphere of white lupin's cluster roots (juvenile, mature and senescent). We also assessed the eliciting capacity of four collection fungi with potential pathogenic impact on white lupin: *Fusarium monoliforme*, *Fusarium solani*, *Pythium ultimum* and *Rhizoctonia solani*. *Fusarium* species are well-known pathogens of white lupin (Bateman, 1997; Raza et al., 2000; Satyaprasad et al., 2000; Shield et al., 2000) and *Pythium ultimum* and *Rhizoctonia solani* also have been reported to infect white lupin (Kuznia et al., 1993; Leach and Clapham, 1992). Moreover, *Rhizoctonia solani* is one of the main pathogens of *Lupinus angustifolius*, the narrow-leaf lupin (MacLeod, 1997).

The aim of this experiment was to get a preliminary answer to the question whether bacterial and fungal elicitation would induce changes in the pattern of secreted phenolics in white lupin seedlings. The experiment was performed a single time (without replicates) and gave interesting first results, which should be confirmed in additional investigations.

2.4.3.3. Material and methods

Seed sterilization

Seeds of *Lupinus albus* L. cv. Amiga (Südwestdeutsche Saatzucht, Rastatt, Germany) were surface-sterilized by incubation in saturated (2.5 %) sodium hypochlorite solution for 30 minutes, and subsequent rinsing with sterile water.

Bacterial elicitation

Four different strains were used for the bacterial elicitation experiments: one strain, previously isolated from the endorhizosphere of juvenile cluster roots (see chapter 3.2.

for more details), was selected for its plant growth promoting abilities (auxin production, phosphate and iron solubilization) and called for this reason "PGPR". Another strain, also isolated during the experiment described in chapter 3.2., but from a bulk soil sample, did not show any PGPR activities and was thus called "neutral". We also used two collection strains, *Escherichia coli*, as a non pathogenic bacteria, and *Pseudomonas syringae* as a potential plant pathogen. White lupin seeds were inoculated by soaking for 15 minutes in a suspension of the corresponding bacterial strain. In addition, we used an autoclaved suspension of *E. coli* to be able to discriminate between the effect of living bacteria and bacterial cell constituents. For negative control, the seeds were soaked in sterile water. After inoculation, seeds were allowed to germinate on plates with a 1 % agar gel. They were harvested five days later.

Fungal elicitation

For fungal elicitation, we used isolated strains from the endorhizosphere of juvenile and senescent cluster roots, as well as from the rhizosphere soil of mature cluster roots. In addition, we used three collection strains, kindly provided by Prof. Geneviève Défago (ETH, Zürich): *Fusarium monoliforme*, *Pythium ultimum* and *Rhizoctonia solani*, three potential plant pathogens. Since the growth of some fungi was very fast, white lupin seeds were first placed on plates containing potato-dextrose-agar (PDA) medium with 1 % agar concentration and allowed to germinate for three days. On the fourth day, fungi were inoculated at the center of each plate. Seedlings were harvested either 2 days later (for fast growing fungi, in our case the collection fungi) or 8 days later. For both incubation times, a negative control was used (seed germination without fungal inoculation).

Harvest of seedlings and collection of exudates

For both fungal and bacterial elicited seedlings, one centimeter was cut from the tip, rinsed in sterile water, weighted and incubated for one hour at room temperature under

gentle shaking to allow collection of exudates. Seedlings were then removed and the water containing the exudates was frozen at -80°C and freeze-dried.

Extraction of phenolics and HPLC analysis

After freeze-drying, samples were extracted with MeOH 80 % as described in the previous chapter (2.2.2.). They were resuspended according to the fresh weight in the first HPLC solvent. HPLC column, solvents and eluting conditions were the same as previously described for the characterization of isoflavonoids in the different stages of cluster roots (see chapter 2.2.2.).

2.4.3.4. Results

The following results are derived from a single study (without replicates), they give interesting tendencies but they have to be interpreted with caution until they receive confirmation through additional experiments.

Secretion of isoflavonoids upon bacterial elicitation

The HPLC profiles for germinating seeds which were incubated in sterile conditions (negative control) were qualitatively different from those obtained previously for roots (see chapter 2.2.2.): genistein (retention time, RT: 28 min) was the most important compound here and malonyl-genistin (RT: 20 min), which was by far dominating the profile in roots, only was found as a minor peak at this early stage of seed germination.

Figure 36

HPLC profiles of secreted phenolics by the apex of five days old white lupin seedlings

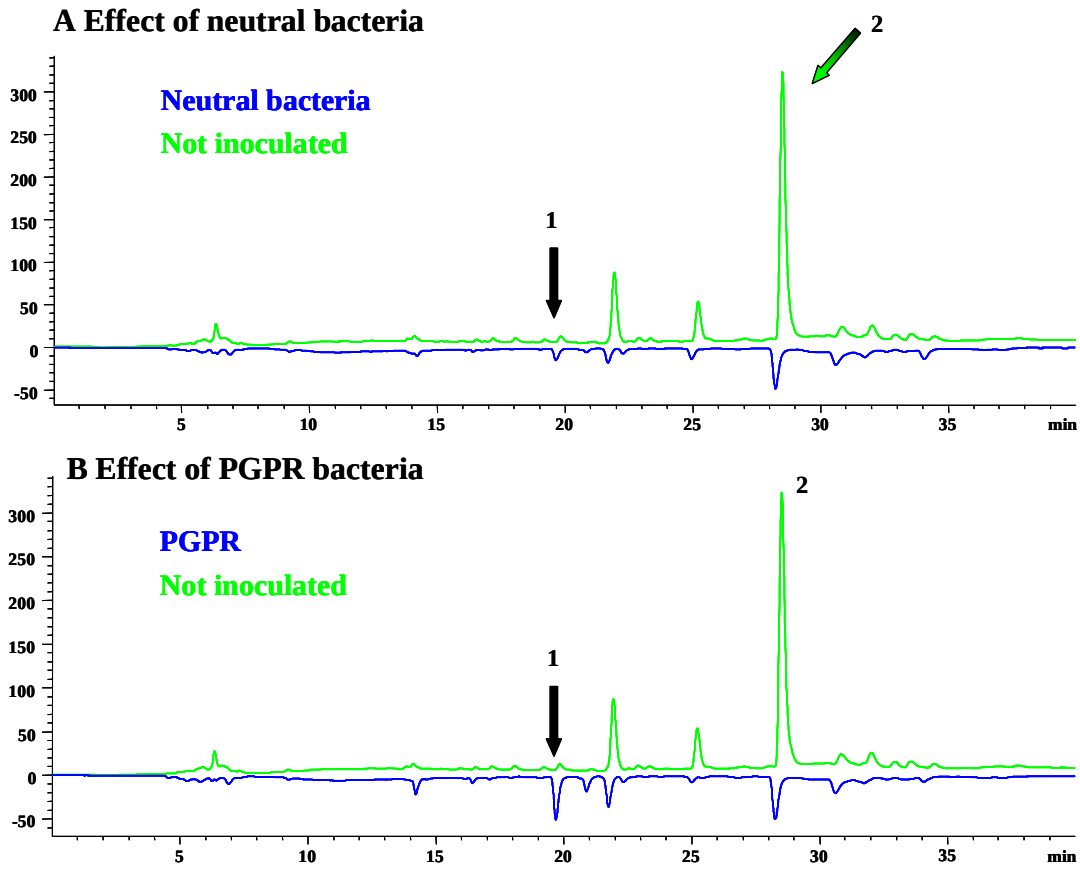


Figure 36: HPLC profiles of secreted phenolics by the apex of five days old white lupin seedlings.

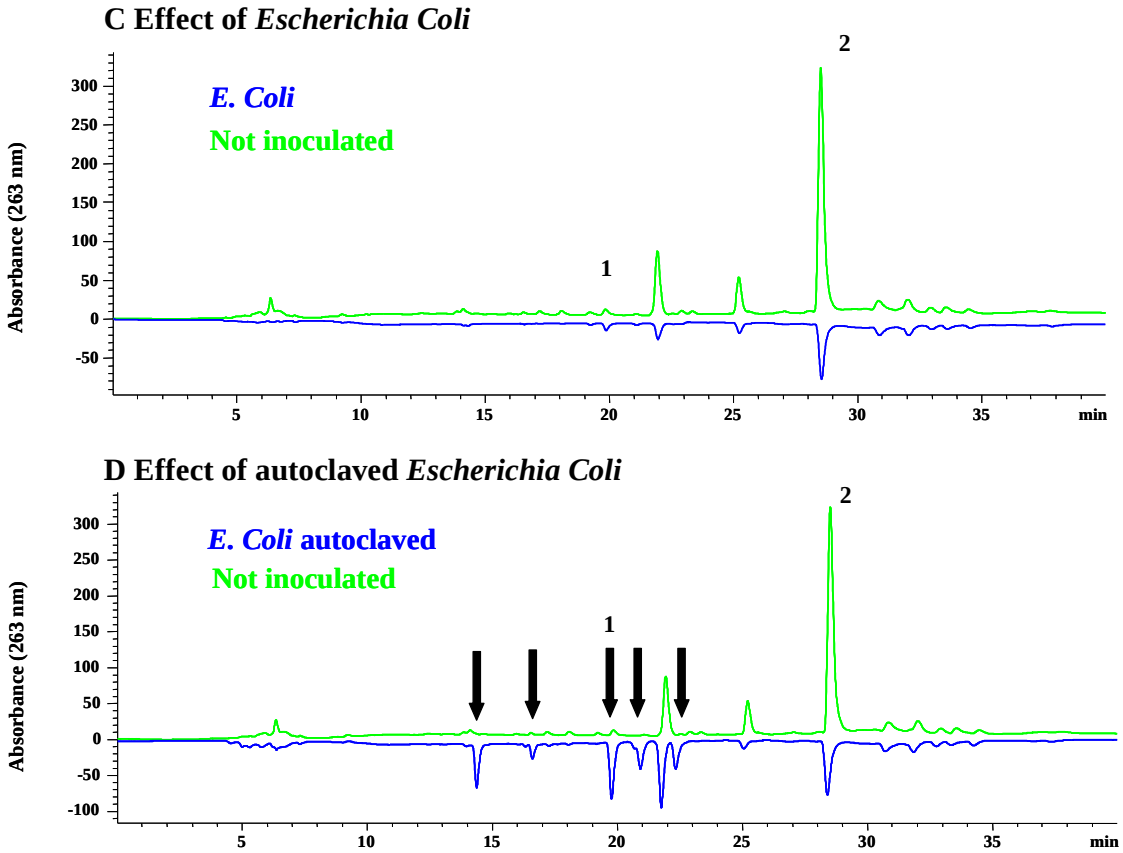
Prior to germination, surface-sterilized seeds were inoculated with a suspension of a neutral bacterium isolated from bulk soil (A), a PGPR bacteria isolated from juvenile cluster roots (B), Escherichia coli. *1: malonyl-genistin; 2: genistein. Absorbance was monitored at 263 nm.*

Inoculating the seeds with bacteria isolated from the rhizosphere of white lupin's roots or from the distant soil had the same effect on the pattern of isoflavonoids recovered (Figure 36 A and B): a general decrease of the phenolics, and especially of genistein, was observed in comparison with seeds which were not inoculated with microorganisms (control). This could be due to a bacterial degradation of the phenolic compounds

secreted during the hour of incubation or to a decrease in secretion caused by the presence of the bacteria. In contrast to the neutral strain, the PGPR strain induced a higher secretion of malonyl-genistein, as indicated by a black arrow in the profile. No specific effect was observed for *Escherichia coli* (Figure 36 C), except for the general decrease also observed previously for the isolated strains. The autoclaved *E. coli* treatment gave an interesting result: the decrease observed on a general level, and in particular for genistein, also was observed in the case of autoclaved bacteria, suggesting that bacterial consumption is, if happening at all, not the only reason for the observed isoflavonoid decrease in the seedling exudates. In addition, some compounds were markedly increased (black arrows) by the treatment with autoclaved *E. coli*, confirming the elicitation role of bacterial components in the secretion of phenolics.

Figure 36 (cont.):

HPLC profiles of phenolics secreted by white lupin seedlings after bacterial elicitation



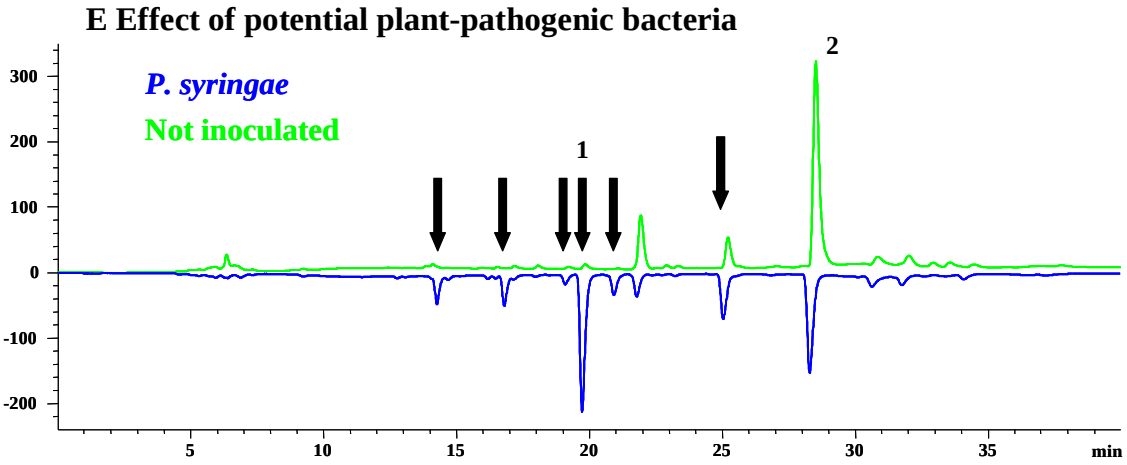


Figure 36 (cont.): HPLC profiles of secreted phenolics by the apex of five days old white lupin seedlings.

Prior to germination, surface-sterilized seeds were inoculated with a suspension of Escherichia coli.*(C), autoclaved* Echerichia coli *(D) and* Pseudomonas syringae *(E). Negative controls are seedlings which were germinated without prior inoculation. 1: malonyl-genistin; 2: genistein. Absorbance was monitored at 263 nm.*

The increased secretion of phenolics was even stronger when a potentially plant-pathogenic bacterium was used to inoculate seeds: the profile for *Pseudomonas syringae* (Figure 36 E) was characterized by increased levels of six different isoflavonoids, suggesting that seedlings reacted differently towards a PGPR or neutral bacteria and towards a potential pathogen.

Secretion of isoflavonoids upon fungal elicitation

The secretion of phenolics after treatment with fungal strains isolated from the rhizosphere of white lupin is shown in Figure 37 A, B and C. While the two isolates recovered from the root fraction of juvenile (A) and senescent (B) cluster roots did not cause big changes in the secretion profile of white lupin seedlings, a very pronounced increase of secretion occurred when seedlings were inoculated with a fungal strain isolated from the rhizosphere soil of mature cluster roots (C). This general increase of a factor of 10 was most marked for one compound (RT: 25 min, black arrow), while genistein was the only isoflavonoid (green arrow) which was not affected by the inoculation.

Figure 37:

HPLC profiles of phenolics secreted by white lupin seedlings after fungal elicitation

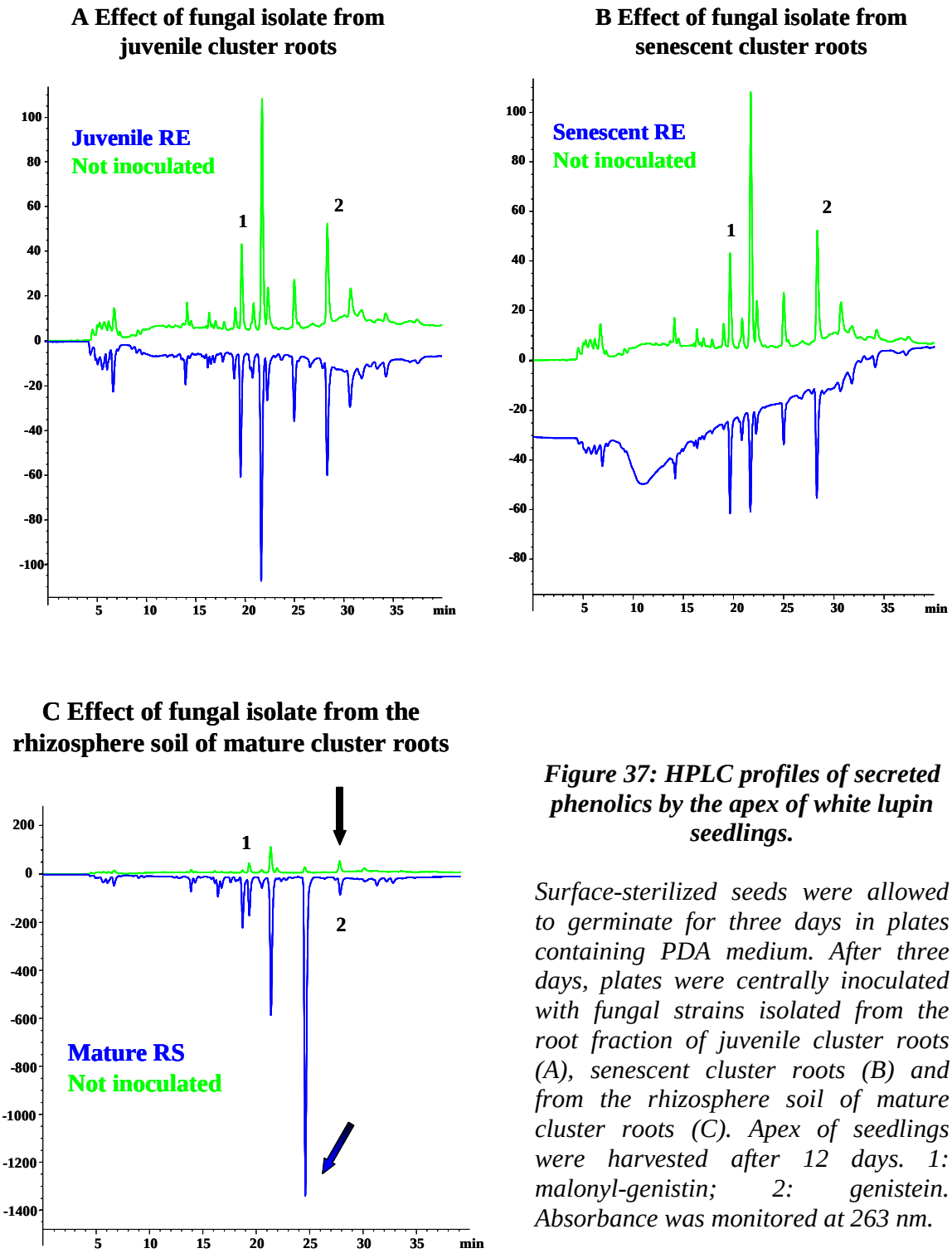


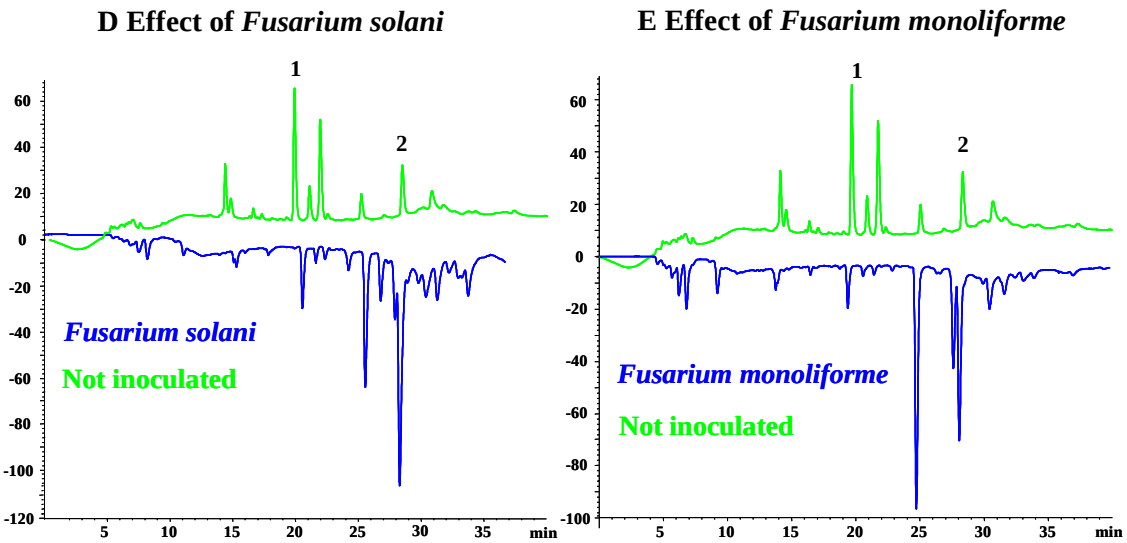
Figure 37: HPLC profiles of secreted phenolics by the apex of white lupin seedlings.

Surface-sterilized seeds were allowed to germinate for three days in plates containing PDA medium. After three days, plates were centrally inoculated with fungal strains isolated from the root fraction of juvenile cluster roots (A), senescent cluster roots (B) and from the rhizosphere soil of mature cluster roots (C). Apex of seedlings were harvested after 12 days. 1: malonyl-genistin; 2: genistein. Absorbance was monitored at 263 nm.

When considering collection strains (Figure 37 D to G), the response was highly variable and strain-dependent. A similar behavior was observed for the two *Fusarium* species (D and E), which induced changes in the last part of the profile, and especially an increased secretion of the compound eluting at a RT of 25 min (see above, Figure 37 C), as well as of genistein, while malonyl-genistin (RT = 20 min) and the two following compounds were decreased in the presence of the elicitor.

Figure 37(cont.):

HPLC profiles of phenolics secreted by white lupin seedlings after fungal elicitation



The highest induction of phenolic secretion was obtained with *P. ultimum* (D), where especially malonyl-genistin, the peak eluting at 25 min, as well as genistein showed an enhanced secretion. As for *R. solani*, no changes were observed when comparing inoculated and non inoculated white lupin seedlings' phenolic secretion.

Figure 37(cont.):

HPLC profiles of phenolics secreted by white lupin seedlings after fungal elicitation

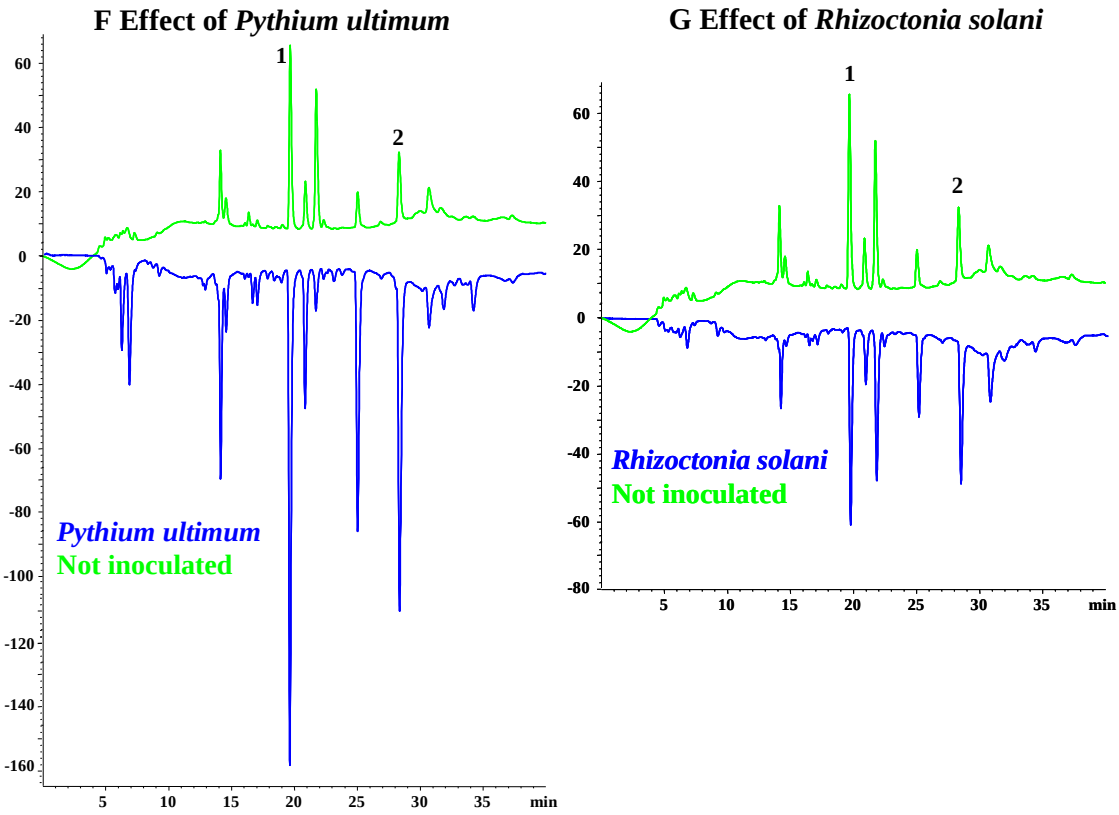


Figure 37(cont.): HPLC profiles of secreted phenolics by the apex of white lupin seedlings.

Surface-sterilized seeds were allowed to germinate for three days in plates containing PDA medium. After three days, plates were centrally inoculated with fungal collection strains: Fusarium solani (D), Fusarium monoliforme (E), Pythium ultimum (F) and Rhizoctonia solani (G). Apices of seedlings were harvested after 6 days. 1: malonyl-genistin; 2: genistein. Absorbance was monitored at 263 nm.

2.4.3.5. Discussion

This work was conducted to investigate a possible role of bacteria and fungi in the elicitation of phenolic secretion in white lupin seedlings. A first interesting result was that the profiles for seedlings differed quantitatively from those obtained from roots, especially with respect to the proportion of malonyl-genistin (RT = 20), which was present as the major compound in roots and genistein (RT = 28), which was the most highly secreted isoflavonoid in young seedlings (Figure 36). Surprisingly, the isoflavonoid profiles for the two kinds of negative controls (bacteria, Figure 36 and fungi, Figure 37) were quite different from each other. This could be due to the age of seedlings, which was ranging from 5 days to 8 days, but was more probably caused by the different germination media: a simple agar gel for bacteria and a rich medium (PDA) for fungi.

As expected, changes between inoculated seeds and negative controls could be observed in most cases. For the two isolated bacteria (Figure 36 A and B), as well as for *E. coli* (C), the presence of the elicitor tended to lower the amounts of secreted phenolics, or to be more accurate, the amounts of phenolics recovered after the hour of incubation. Isoflavonoid degradation by bacteria remaining on the seedlings despite the washing step cannot be excluded (Barz, 1970; Cooper and Rao, 1995), even if the treatment with autoclaved *E. coli* (D) yielded as little genistein as the other treatments (A and B). This decreased genistein secretion upon elicitation with autoclaved *E. coli* could be the result of an elicitor-induced reduction of the production or secretion, or simply the consequence of an intrinsic balance between the different isoflavonoids produced and secreted in white lupin seedlings, decreasing some of them while others are secreted in higher amounts. The increased levels of five isoflavonoids in the profile of seedlings elicited with autoclaved *E. coli* treatment confirmed the elicitor role of bacterial components. The fact that these compounds were not increased, and for some even decreased, by the treatment with living *E. coli* can be explained in two different ways: on the one hand, the secretion of these compounds might also have been increased in the living *E. coli* treatment, but reduced again by a bacterial degradation during the exudate collection time and on the other hand, the eliciting molecules might be intracellular constituents or cell wall

fragments, liberated during the autoclaving step. The profile obtained after inoculation with *Pseudomonas syringae* (Figure 36 E) showed large differences when compared with the three other inoculated bacteria (Figure 36 A, B and C): six compounds were recovered in higher amounts than in the non inoculated control, among which malonyl-genistin (RT: 20 min). This result indicates that white lupin seedlings react differently towards different bacteria, with one common behavior in the case of neutral or potentially beneficial bacteria (Figure 36 A, B and C) and a very different behavior for a potentially deleterious bacterium (E). It would have been interesting to test the effect of a nitrogen-fixing bacterium as elicitor, since flavonoids and isoflavonoids are known to play a role in the establishment of the nitrogen fixing symbiosis in legumes. One can thus assume that other isoflavonoids would have been elicited with a rhizobium or a bradyrhizobium strain.

The elicitation experiment with fungi gave different results (Figure 37): in most cases, no significant difference in secreted phenolics was observed for the seedlings germinated in presence of fungi, in comparison with the negative control. While the fungal strain isolated from the root fraction of juvenile (A) cluster roots did not induce any changes, the one isolated from the senescent (B) cluster roots caused reduced levels of a single compound (RT: 22 min). The situation was quite different with the fungus isolated from the rhizosphere soil of mature cluster roots: a drastic increase of phenolic secretion was induced in this case (Figure 37 C). All compounds except genistein were increased, with a marked effect for one of them, eluting at 25 min. This very interesting effect would deserve further investigations: a study of many replicates to see if it is reproducible, as well as an identification of the strain and an elucidation of its potential pathogenic effect. It would also be interesting to test rhizosphere isolates on a larger scale, to see if the rhizosphere of mature cluster roots differs from the rhizosphere of other cluster root stages, with respect to the proportion of fungal strains eliciting such a response.

The effect of the fungal collection strains was dependent on the species considered: interestingly, both *Fusarium* species (Figure 37 D and E) gave a similar response, with reduced levels of phenolics in the first part of the profile (more hydrophilic compounds)

and enhanced levels in the end of the profile. Especially genistein was found in increased levels in both *Fusarium* species, while the compound eluting at 25 min was much more increased in the case of *F. moniliforme*, as already observed for the strain isolated form the rhizosphere soil of mature cluster roots (see also Figure 37 C). The two other fungi (F and G) caused different responses in the secretion of phenolics by white lupin seedlings: *Rhizoctonia solani* had no effect at all, but *Pythium ultimum* induced an increase in all compounds but one (RT: 22 min). This is the only one of the four collection strains which caused a higher accumulation of malonyl-genistin (RT: 20 min), similar as was observed for the strain isolated from the rhizosphere of mature cluster roots. All of these collection fungi have been reported to infect white lupin and as can be deduced from the Figure 2, they cause different changes in the profiles of secreted isoflavonoids in white lupin seedlings. Provided these differences would be reproducible in additional experiments, they would also deserve further investigations. In particular, the elucidation of the molecular structure of the isoflavonoid eluting at 25 min would be very interesting. This compound was not only highly increased upon treatment with the mature cluster root fungal strain, but also after inoculation by *F. monoliforme, P. utlimum* and, to a lesser extent, by *F. solani* and *P. syringae* and since it seems to have a broad range of elicitors, it might play a role in the general plant defense.

In conclusion, this experiment provided interesting preliminary results and a good start for additional experiments that should enable us to better understand the chemical communication occurring between white lupin and rhizosphere bacteria and fungi. An improved experimental setup would take more replicates into consideration, analyze a larger number of isolated bacteria and fungi, and allow a separation of the "elicitation" and "degradation" processes, for instance through the inhibition of microbial growth during the collection of exudates.

2.4.4. Synthesis

2.4.4.1. Characterization of the major isoflavonoids in white lupin cluster roots

In this chapter, the contents and secretion of isoflavonoids were investigated in different stages of cluster root development. Using a LC-MS approach, twelve major compounds were identified, with genistein and hydroxygenistein and their corresponding mono- or diglycosylated conjugates. The compound dominating the HPLC profile, identified as genistein 6"-*O*-malonyl-O-glucoside, was found to be less abundant in terms of weight as genistein, which was by far the isoflavonoid secreted in highest amounts in white lupin cluster roots (approx. 700 µg . g root FW . h^{-1}, as compared with 40 µg . root FW . h^{-1}). Genistein 6"-*O*-malonyl-O-glucoside had a much higher UV absorbing capacity than genistein.

The amounts of secreted isoflavonoids by white lupin were significantly increased upon P deficiency, as previously reported in two other leguminous species, soybean (Murali et al., 1983) and bean (Juszczuk et al., 2004). Moreover, isoflavonoid secretion was slightly but still significantly higher in cluster roots than in non cluster roots, especially for genistein, which is in agreement with what Neumann and coworkers observed in 2000 for phenolics in general. In my eyes, the most interesting result of this study was the pattern of secretion along growing cluster roots: while up to now, since studies had been focused mainly on organic acid secretion, it was believed that most of the secretion processes were taking place at the mature stage of cluster roots, we showed that for isoflavonoids, the peak of secretion occurred at earlier stages, in young and immature cluster roots. White lupin cluster roots thus appeared to share out the tasks, with younger roots secreting isoflavonoids and mature roots secreting organic acids. This pattern of secretion was globally the same for all major compounds. However, the ratio between secreted and internal isoflavonoid amounts was different, with the highest ratio (secreted *vs.* internal) for genistein, indicating a high secretion which can be related to the fact that this aglycone has a less hydrophilic structure than all other identified isoflavonoids.

2.4.4.2. Effect of microbial elicitation on the isoflavonoid secretion in white lupin seedlings

To assess the question of bacterial and fungal elicitation of isoflavonoid secretion, we used white lupin seedlings. We tested the effect of several isolates coming from the rhizosphere of white lupin, as well as the effect of collection strains. This experiment was performed as a preliminary test to see if differences could be observed between elicited and non elicited white lupin seedlings and it should be repeated to confirm the results obtained, which were based on a single experiment without replicates.

We observed that when white lupin seeds were inoculated with either "neutral", or PGPR (plant growth promoting rhizobacteria) bacteria, secretion of phenolics by the seedlings decreased. In contrast, using potentially pathogenic bacteria induced a higher secretion of isoflavonoids, which indicated that white lupin seedlings reacted in the opposite way when challenged with either potentially beneficial or potentially deleterious bacteria. Interestingly, while seed inoculation with a suspension of *E. coli* caused a decrease in phenolics, inoculation with the corresponding autoclaved suspension drastically increased secretion of phenolics, suggesting that "elicitor signals" were liberated through the cell lyses occurring during autoclaving.

As for fungi, the reactions in phenolic secretion by the seedlings were strain specific: fungi isolated from juvenile and senescent cluster roots did not alter the profile of secreted phenolics. In contrast, the fungal strain isolated from mature cluster roots caused a drastic increase of the isoflavonoid secreted, suggesting a strong reaction of the seedling. Collection strains corresponding to potential pathogens of white lupin induced the following reactions: *Fusarium solani* and *Fusarium monoliforme* caused similar changes, with decreased levels of the more hydrophilic isoflavonoids and an increased in the less hydrophilic ones, especially genistein. *Rhizoctonia solani* did not induce any changes in the profile of secreted phenolics, in contrast to *Pythium ultimum*, which induced an increased secretion of many isoflavonoids.

In conclusion, the secretion of phenolics by white lupin seedlings was affected differentially by bacterial and fungal strains and it would be worthwhile to repeat the experiment with replicates to see if these interesting preliminary results are reproducible.

2.4.5. Discussion – Outlook

Thanks to a fruitful collaboration with chemists from the University of Neuchâtel (Dr. E. Abou-Mansour and Prof. R. Tabacchi), we were able to identify the isoflavonoids secreted by different stages of white lupin cluster roots. Investigating secreted compounds in addition to internal contents of roots proved to be a good idea, since differences in isoflavonoids with respect to P supply, root type (cluster *vs.* non cluster) and cluster root stages were mainly visible in the secretion patterns, while the internal contents showed only little variations. Moreover, since we were interested in the impact of isoflavonoids in plant-microbe interactions, it made sense to analyze the compounds which are secreted into the rhizosphere and might play a role there, in addition to the internal root isoflavonoid contents. From our results, it appears that cluster roots taken as a whole (all stages together) are not as different from non cluster roots for isoflavonoid as for organic acid secretion. However, it has been previously demonstrated for organic acids that cluster roots cannot be considered as homogeneous structures, but that stages differ very much from each other with respect to their secretion behavior. Until now, root secretion had been described to be highest in mature roots, because studies focused on organic acids (Massonneau et al., 2001; Neumann et al., 2000), but as we observed in the present study, root secretion as a general phenomenon is not restricted to mature roots, since isoflavonoids are secreted mostly at the juvenile and immature stages. Thus, comparing whole cluster roots with non cluster roots does not take into account cluster root heterogeneity and could lead to deceptive conclusions. In our case, we could observe that even if, on the whole, only slight quantitative differences were observed between cluster and non cluster roots, juvenile and immature cluster roots secreted much more isoflavonoids than normal roots. On the other hand, the age of non cluster roots should also be considered for proper comparisons: from our *in situ* staining of flavonoids in the whole lupin root system (see chapter 3.5.), we can clearly see that in non cluster roots also, flavonoid secretion is age dependent, with a higher secretion in the apices and young roots.

The higher secretion of flavonoids in juvenile and immature cluster roots can be linked to the findings of Langlade and coworkers (2002), who studied the expression levels and activities of ATP-citrate-lyase (ACL, see also chapter 2.2.2.) and found highest expression and activity in juvenile cluster roots. ACL is responsible for the transformation of citrate into oxaloacetate and acetyl-CoA. This enhanced activity in juvenile roots could furnish the supply of acetyl-CoA for the flavonoid biosynthetic pathway, in addition to the supply for the synthesis of membrane lipids.

Since isoflavonoids are known to be involved in plant microbe interactions, not only in the attraction of symbiotically nitrogen-fixing bacteria, but also in defense mechanisms against bacterial and fungal pathogens, we investigated the effect of these compounds on microbial growth (see chapter 3.5.), as well as the effect of microbes on isoflavonoid secretion. For the effect of microorganisms on the secretion of isoflavonoids, our preliminary results indicated that white lupin reacted differently towards "neutral" and PGPR bacteria, with a decrease of phenolic secretion, or towards potentially pathogenic bacteria, with an increase of phenolic secretion. This would support the previously described role of isoflavonoids as phytoalexins. Interestingly, there was a strong reaction (higher phenolic secretion) of white lupin seedlings to a fungal strain isolated from mature cluster roots, while no effect was observed for the strains coming from juvenile or senescent cluster roots. If this effect is reproducible, it would be interesting to identify this strain and to characterize it with respect to pathogenicity and / or ability to metabolize citrate. We could observe that depending on the fungal strain used to elicit isoflavonoid secretion, different compounds were secreted in increased amounts, indicating a species specific reaction. Again, if these results can be reproduced, it would be interesting to isolate the differentially secreted compounds and to test them for antimicrobial activity.

2.5. SYNTHESIS

Throughout this chapter, we analyzed several aspects of root secretion physiology and phosphate acquisition by white lupin cluster roots.

Langlade et al. (2002) observed a correlation between malate secretion and the activity of ATP citrate lyase in growing cluster roots of white lupin, which led to postulate that ACL was involved in organic acid metabolism, in addition to its role in lipid and phenolic biosynthesis. To verify this putative role, we overexpressed the lupin ACL in *A. thaliana* and analyzed the secretion of organic acids and phenolic compounds. Our results gave preliminary indications that in P-deficient conditions, ACL indeed might be involved in organic acid metabolism, since higher amounts of malate were secreted from the overexpressing plants than from the corresponding wild-type. Moreover, the observation of higher amounts of two phenolic compounds in the exudates of ACL overexpressing plants also indicated a possible link between the high ACL activity and the enhanced secretion of phenolics observed in white lupin juvenile cluster roots. However, further investigations including more lines and replicates, as well as a better cultivation system should be performed to really be able to conclude about the role of ACL in organic acid or phenolic metabolism.

Going from the investigation of the molecular processes leading to the enhanced organic acid secretion to the role of these organic acids in phosphate acquisition, we performed a microcosm study to monitor P dynamics at different root proximity levels and over a one year cultivation period. This experiment was designed to come as close as possible to natural conditions and interestingly, most of the processes described in hydroponic systems could be verified with our soil-grown plants. We could show that over a one year cultivation period, important desorption processes took place in the rhizosphere of white lupin and that most of this desorption, as well as most of the phosphatase activity and organic acid secretion occurred in the rhizosphere of cluster roots, while the same processes took place in non cluster roots, but to a much lesser extent. Significant differences were found for all

parameters studied between the rhizosphere soil of cluster and non cluster roots, despite the fact that the cluster root stages could not be separated and that only a little part of the root harvested as "cluster roots" corresponded to active cluster roots.

In addition to the secretion of organic acids, we could show in the last part of this chapter that white lupin cluster roots also secreted considerable amounts of phenolic compounds (up to 700 µg of genistein . g root FW . h^{-1}), most of them belonging to the isoflavonoid family. P deficiency induced a higher secretion of isoflavonoids, especially genistein and isoflavonoids were generally secreted in higher amounts by cluster roots than by non cluster roots. Most of the secretion took place at the young and immature stages of white lupin cluster roots, prior to the stage of citrate secretion. This indicated a sharing out of the tasks in growing cluster roots and the fact that isoflavonoids were secreted prior to citrate and not afterwards led us to think that these compounds might play a role in inhibiting microbial degradation of citrate (see also chapter 3 for this topic). A clear effect could be seen when white lupin seedlings were germinated in contact with bacterial or fungal strains, with respect to the secretion of isoflavonoids. Depending on the strain, phenolic secretion was either reduced or highly increased, indicating a species specific reaction from white lupin seedlings. Strongest reactions (increased secretion of phenolics) were obtained upon elicitation with an autoclaved suspension of *E. coli* as well as with a suspension of living *P. syringae* for bacteria and with a fungal strain isolated from the rhizosphere of white lupin mature cluster roots for fungi.

2.6. DISCUSSION – OUTLOOK

The topics investigated in this chapter obviously are very diverse and may seem, at first sight, quite unconnected. However, they all have the "cluster root theme" in common, and reflect the interdisciplinary approach we used to obtain a picture as complete as a possible of cluster root functioning. We went through the analysis of molecular control of the secretion processes (ACL), the secretion processes themselves (isoflavonoids), finally to the consequence of this secretion for the plant's nutrition (P dynamics).

Even if we could not bring a definitive answer to the question of ACL's involvement in the malate/citrate shift in cluster roots, we learned many things through these experiments: i) the whole methodological procedure of genetic manipulation, from the cloning of the gene of interest to the obtaining of transformed plants, ii) the importance of using many different lines and replicates in transgenic studies and iii) the complexity of gene expression manipulation and the necessity to find suitable and inducible promoters for the study of basic plant metabolic functions. Despite the theoretical simplicity of such a genetic approach to study the function of an enzyme, the results obtained were not univocal and this made us realize that processes occurring in a cell or even in a plant are much more complex that what we expect and what we presently can control. Overexpressing a gene under the control of a constitutive promoter may either be inefficient due to a plant mechanism to protect itself against this disturbance (like silencing of overexpressed genes, which might well have happened in our case with ACL) or be so drastic that many side-effects are generated, which makes it difficult to understand the phenotype and to come to final conclusions about the functions of the gene of interest.

The characterization of the pattern of isoflavonoids produced in cluster roots and secreted from these roots allowed to show that cluster root secretion was not restricted to the well known organic acids, but that phenolics also were secreted in significant amounts. Isoflavonoids might play several roles in white lupin's rhizosphere: first, they might act, as will be discussed also in chapter 3, as antimicrobial compounds, which would limit the

microbial degradation of citrate. Clearly the fact that they are secreted in highest amounts just before citrate secretion is a hint that they might inhibit microbial growth or exert a repulsive effect (negative chemotaxism), which would induce mobile rhizobacteria to move away from roots. However, we must admit that we could not verify these hypotheses experimentally through the *in vitro* tests performed, where no negative effects on bacteria and only slight effects on fungi could be observed. Another possible function of isoflavonoids is the solubilization of iron through chelation. It has been previously reported that isoflavonoids can act as siderophores (Moran et al. 1997; Zhang et al., 1991; Römheld and Marschner, 1983) and even if white lupins, as dicotyledonous plants, may rely principally on reductases for iron acquisition (Römheld and Marschner, 1981), it cannot be excluded that isoflavonoids may also contribute to solubilization. Moreover, even in the case of a reductase-mediated iron acquisition, Fe III has to be solubilized through acidification and chelation in order to become accessible to the reductases (Curie and Briat, 2003). It would be interesting to compare the pattern of secreted isoflavonoids in cluster roots of iron deficient plants with the pattern obtained here in the case of phosphate deficiency. Such studies comparing cluster root structure and secretion in iron- or phosphate deficient conditions have been conducted already in white lupin (Hägstrom et al., 2001; McCluskey et al., 2004) but they did not include isoflavonoid analysis. Hägstrom et al. (2001) observed that more phenolics were produced in the case of iron deficiency, but this was only deduced from a phenolic specific staining and no information on the chemical structure of these phenolic compounds was presented. In addition to the potential iron-solubilizing and antimicrobial effects, isoflavonoids such as genistein (Baumberger et al., 2003; Duzan et al., 2004) and daidzein (Abd-Alla, 2001; Sharma et al., 2002) are also known to attract symbiotically fixing nitrogen bacteria (ref) and this might be effectively taking place in a leguminous plant such as white lupin. Our experiments were conducted in nitrogen sufficient conditions and as for iron, an interesting complementary experiment would be to analyze the isoflavonoid secretion in plants grown in nitrogen deficient conditions.

Finally, coming to the effect of these root exudates, and this time mostly organic acids and phosphatases, on phosphorus acquisition in white lupin, we observed that after six

generations of continuously cropped lupin, approximately 2 % of the soil phosphate had been transferred to the shoots. This uptake of phosphate mainly took place in the vicinity of cluster roots, as indicated by the higher i) P desorption, ii) citrate contents and iii) phosphatase activities in the rhizosphere soil of cluster roots. This experiment, which was performed in close collaboration with Dr. C. Le Bayon (University of Neuchâtel) added a substantial aspect to the present work, in the sense that it investigated P acquisition, as the final consequence of cluster root formation and secretion physiology. The experimental setup which came quite close to real field conditions also offered an important complement to the other experiments, which were mostly performed in hydroponic cultures or in microcosms with inoculated sand (see chapter 3, Plant microbe interactions).

In conclusion, we believe that the results presented in this section contributed to the better understanding of cluster root secretion physiology, from the molecular control of organic acid secretion to the analysis of secondary compounds and finally to the impact of cluster roots and their battery of secreted organic compounds on phosphorus acquisition by soil-grown lupins.

CHAPTER III

Plant microbe interactions in the rhizosphere of Lupinus albus and Arabidopsis thaliana

Chapter III: overview

Chapter III: overview (continued)

3.1. INTRODUCTION

This third chapter is devoted to the study of plant microbe interactions in the rhizosphere of white lupin cluster roots. We investigated the abundance, diversity, function and community structure of the bacterial communities (chapter 3.2.), as well as the diversity of potential associative nitrogen fixers (chapter 3.3.) associated with white lupin's cluster roots. Moreover, the elucidation of white lupin's secreted isoflavonoids (chapter 2.4.) led to the question of the possible impact of such phenolic compounds on the structuring of rhizosphere bacterial communities. Since no mutants are available in white lupin, we decided to address this question in *Arabidopsis thaliana* and used a chalcone synthase-impaired mutant to investigate the changes between mutant and wild-type in the respective associated bacterial communities (chapter 3.4.). The last part of this chapter on plant microbe interactions deals with potential protection mechanisms evolved by white lupin against microbial degradation of the phosphate-solubilizing agents, citrate and malate (chapter 3.5.).

3.1.1. Potential plant microbe interactions in the rhizosphere

Soil microorganisms are key players in the soil-plant system and their roles in plant nutrition, health and protection have often been demonstrated in the past decades. In addition to their general role in nutrient cycling and mineralization of the soil organic matter, they also interact more directly with plants in various ways. Both microorganisms and plants depend on the soil mineral nutrients. However, microorganisms are much more efficient than plants in the uptake of these elements. Most plants rely on mycorrhizal fungi for their mineral nutriment acquisition and it has been shown as well that bacteria also, in addition to filling their own needs, can supply plants with phosphate by solubilization through secretion of protons (Ca P complexes) or carboxylates (Fe or Al P complexes) (Chabot et al., 1996; Gyaneshwar et al., 2002; Singh and Kapoor, 1999). In addition, bacteria have been shown to produce siderophores which increase the availability of iron (Chen et al., 1998; Duijff et al., 1994; Sharma et al., 2003). A different

kind of relationship is observed for organic compounds: plants are releasing a large amount of them through the root exudates, and these root-secreted organic molecules can then be used as a carbon source by heterotrophic rhizosphere microorganisms. This organic carbon flux through the roots is one of the main reasons why the rhizosphere harbors a higher bacterial abundance than the bulk soil. This root: soil ratio (R:S ratio) however was mostly measured with culture-dependent methods and may also reflect a change in cultivability due to a shift in bacterial growth strategy (Aragno, 2005): from the slow growing bacteria (K-strategists) in the bulk soil to the fast-growing ones in the root vicinity (r-strategists). The r-strategists, which are used to growth conditions where nutrients are available in high amounts, will be more easily cultivated in rich culture media. The higher abundance of bacteria in the rhizosphere in comparison to the bulk soil should thus be also tested with culture-independent methods, such as microscopic counts. Furthermore plants also exert a selective effect (Marilley and Aragno, 1999), in addition to the elective effect due to the enrichment in root-exuded organic carbon. This selective effect may be related to the secretion of specific compounds inhibiting the growth of rhizosphere microorganisms or to a change in the chemical and physical conditions of the root environment, for instance in a change of pH or redox status. This general effect of plants on the surrounding microflora is expected to cause on the one hand a higher abundance of bacteria and on the other hand a lower diversity.

In addition to the supply of phosphate or of nitrogen through symbiotic or associative fixation, bacteria can influence the plant growth and health in many ways (Glick, 1995), either directly by producing plant hormones like auxin (Asghar et al., 2002; Patten and Glick, 2002), or indirectly by protecting the plant against potential pathogens through i) production of antibiotics (Haas and Défago, 2005; Pal et al., 2001), ii) degradation of the fungal cell wall (Cattelan et al., 1999; Fogliano et al., 2002), iii) induction of the plant defense mechanisms (Iavicoli et al., 2003; Pieterse et al., 2003) or iv) competition for iron (Lee et al., 2003; Ran et al., 2005). Bacteria which perform one or the other of these mechanisms are called “plant growth promoting rhizobacteria” (PGPR). There also are deleterious bacteria, of course, but most of the plant diseases are caused by pathogenic fungi, like *Fusarium* or *Pythium* species. Both kinds of pathogens, bacteria and fungi,

have received a lot of attention in the field of plant pathology due to the yield losses they cause. However, it is not our aim in this chapter to enter into such investigations. In the first part, we describe the bacterial communities in general, with a particular focus on PGPR activities (chapter 3.2.), nitrogen fixation (chapter 3.3.), effect of phenolics in the structuring of bacterial communities (chapter 3.4.) and only in the last topic (chapter 3.5.) do we address the question of a potentially deleterious effect of bacteria on plant nutrition through the degradation and utilization as a carbon source of the citrate secreted by the plant to acquire phosphate from soils where it is only sparingly available.

3.1.2. The case of white lupin's rhizosphere

In addition to the general rhizosphere characteristics which are mentioned above, white lupin's rhizosphere, and especially the cluster root environment, offers very particular conditions to the surrounding microflora. As already described in chapter 2, white lupin's cluster roots go through different developmental stages, which are characterized by different secretion activities: at the juvenile stage, roots are still growing, they secrete little amounts of malate and high quantities of isoflavonoids. Few days later, cluster roots reach the immature stage, where isoflavonoid secretion is highest, while organic acid secretion is low. The opposite is observed at the mature stage, characterized by the secretion of very high amounts of citrate and a concomitant release of protons causing rhizosphere acidification, while isoflavonoids are not secreted in high quantities. At the senescent stage, the secretion of both isoflavonoids and organic acids decreases strongly. Each cluster root stage is lasting for only few days and this gives an idea of the strong and fast changes which are occurring in the rhizosphere of a growing cluster root: not only in terms of secreted organic compounds, phenolics or organic acids, but also in the physicochemical properties of the rhizosphere, like the pH or the oxygen levels (Neumann et al., 2000), two parameters which are likely to influence the microbial communities.

The precise knowledge of the secretion physiology which characterizes each cluster root growth stage makes white lupin a good model to study rhizosphere plant microbe interactions. Most of the work available on plant microbe interactions in the rhizosphere of white lupin deals with symbiotic nitrogen fixation (Gonzalez-Sama, 2004; Roughley et al., 1993; Trujillo et al., 2005). Very recently, Unno and co-workers (2005) isolated phytate-hydrolyzing bacterial populations from the rhizosphere of white lupin and the sequencing analysis revealed that most of the isolates were classified as *Burkholderia*. A previous report (Balandreau et al., 2001) already mentioned the occurrence of *Burkholderia* species in the rhizosphere of white lupin. But in all these cases, white lupins were grown in P-sufficient conditions and studies dealt with normal roots and not with cluster roots. In contrast, not much attention has been paid so far to the microflora associated with cluster roots, despite the numerous reports on the secretion physiology of these roots. Some earlier reports addressed the potential impact of bacteria in cluster root formation (Gardner et al., 1982a; Lamont and McComb, 1974; Malajcuk and Bowen, 1974), but till now, no clear answer has been given to this question. To our knowledge, only one study analyzed the bacterial communities associated with white lupin's cluster roots with molecular tools (Marschner et al., 2002). They showed that the microflora was influenced by the type of organic acid secreted, the cluster root stage and the plant age. However, this study focused on the community structure and did not describe the microflora from other perspectives. To gain more insights into the plant microbe interactions in white lupin's rhizosphere, we analyzed the abundance, function, diversity and community structure of the bacterial communities associated with white lupin's cluster roots (chapter 3.2.). We also investigated the diversity of potential associative nitrogen fixers (chapter 3.3.) in cluster and non cluster roots of white lupin. Finally, we looked for potential protection mechanisms of white lupin against microbial degradation of the phosphate-chelating agents (chapter 3.5.).

3.1.3. Use of *Arabidopsis thaliana* mutants to study plant microbe interactions in the rhizosphere

One of the shortcomings of the use of white lupin as a model is the fact that the genome of this plant has not yet been elucidated and that it cannot be genetically transformed. No mutants are thus available in white lupin. Since we wanted to investigate the impact of secretion of phenolics on the structuring of rhizosphere bacterial communities, we had to change the model plant and to use *Arabidopsis thaliana* to take advantage of the availability of mutants in this plant. Surprisingly, the first papers on plant microbe interactions in *Arabidopsis* rhizosphere only appear in the nineties. Many focused on plant resistance against pathogens and on the elucidation of the plant's defense pathways (Pieterse et al., 1996, 1998, 2003; Ton et al., 1999; van Wees et al., 1999), but some others were also conducted to investigate the diversity of *Pseudomonas* populations associated with *Arabidopsis* roots (Fromin et al., 2001; Persello-Cartieaux et al., 2001), as well as the root colonization by PGPRs in different mutants of *Arabidopsis* (O'Gallaghan et al., 2001). Since we were interested to know if a change in root secretion of phenolics would have an impact on the root associated bacterial communities, we chose one of the *Arabidopsis transparent testa* mutants, characterized by a null mutation in the chalcone-synthase gene, which encodes the very first enzyme in the flavonoid biosynthetic pathway. This mutant, *tt4*, has been described in the literature as lacking flavonoids in all plant parts (Burbulis et al., 1996) and provided a useful tool to investigate the impact of flavonoids on the structuring of bacterial communities. In chapter 3.4., we used a molecular approach to compare the communities associated with the rhizosphere of the *tt4* mutant lacking flavonoids and with the corresponding wild-type.

3.2. SECRETION ACTIVITY OF WHITE LUPIN'S CLUSTER ROOTS INFLUENCES BACTERIAL ABUNDANCE, FUNCTION AND COMMUNITY STRUCTURE

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3.2.1. Abstract

White lupin (*Lupinus albus* L. cv. Amiga) reacts to phosphate deficiency by producing cluster roots which exude large amounts of organic acids. The detailed knowledge of the secretion physiology of the different root parts makes it a good model plant to study plant-bacteria interaction. Since the effect of the organic acid exudation by cluster roots on the rhizosphere microflora is still poorly understood, we investigated the abundance, diversity and functions of bacteria associated with the cluster roots of white lupin, with special emphasis on the influence of root proximity (comparing root, rhizosphere soil and bulk soil fractions) and cluster root growth stages, which are characterized by different secretion activities. Plants were grown for five weeks in microcosms, in the presence of low phosphate concentrations, on acidic sand inoculated with a soil suspension from a lupin field. Plate counts showed that bacterial abundance decreased at the stage where the cluster root secretes high amounts of citrate and protons. *In vitro* tests on isolates showed that the frequencies of auxin producers were highest in juvenile and mature cluster roots and significantly decreased in senescent cluster roots. However, no significant difference in the frequency of auxin producers was found between cluster and non cluster roots. The diversity and structure of bacterial communities were investigated by DGGE of 16S rDNA and 16S rRNA. The diversity and community structure were mostly influenced by root proximity and, to a lesser extent, by cluster

root stage. The richness of bacterial communities decreased with root proximity, whereas the proportion of active populations increased. The high citrate and proton secretion occurring at the mature stage of cluster roots had a strong impact on the structure and richness of the bacterial communities, both in the root and in the rhizosphere soil.

3.2.2. Introduction

When growing in soils with sparingly available phosphate, plants can display several mechanisms to cope with phosphate deficiency (Raghothama, 1999; Schachtman et al., 1998). One mechanism is the formation of cluster roots, also called proteoid roots (Purnell, 1960), which is linked to the secretion of high amounts of organic acids in the rhizosphere (Lamont, 2003; Neumann and Martinoia, 2002; Roelofs et al., 2001; Veneklaas et al., 2003). Organic acids act as phosphate solubilizers, mainly by acidification in calcareous soils and by chelation and ligand exchange in acidic soils (Dakora and Phillips, 2002; Dinkelaker et al., 1989, 1997; Gerke et al., 1994, 2000; Jones and Darrah, 1994; Ryan et al., 2001). Many studies on cluster root formation and secretion physiology and ecology have been conducted (Keerthisinghe et al., 1998; Lamont, 2003; Neumann and Martinoia 2002; Shane et al., 2003a; Skene, 1998; Watt and Evans, 1999) and have led to a better understanding of this particular adaptation to low-nutrient soils. However, the effect of these roots and their exudates, mainly organic acids and phenolics (Dakora and Phillips, 2002; Dinkelaker et al, 1997; Jung et al., 2003; Neumann et al., 2000; Wojatsek et al., 1993), on the rhizosphere microflora has been studied far less. Some studies have been conducted to investigate the potential role of bacteria in cluster root formation (Gardner et al., 1982a; Lamont and McComb, 1974; Lamont et al., 1984; Malajcuk and Bowen, 1974). They showed that cluster roots can be produced in sterile conditions, but that their formation is enhanced by the presence of bacteria.

Tools to obtain a closer insight into plant-bacteria interactions have recently been developed. The availability of powerful fingerprinting methods based on 16S rDNA and rRNA polymorphism now makes it possible to compare the microbial communities associated with cluster and non cluster roots in a culture-independent manner. To our knowledge, only one study (Marschner et al., 2002) has used molecular tools to assess the structure of microbial communities in the rhizosphere of a cluster-rooted species, white lupin (*Lupinus albus*). They showed that the microflora is influenced by the type of organic acid secreted, the cluster root stage and the plant age.

Lupinus albus is the sole cluster-rooted species of agricultural importance and offers many advantages as a model plant: (i) fast growth, (ii) high root biomass and (iii) good knowledge of secretion physiology (Gardner et al., 1982b, 1982c, 1983; Johnson et al., 1994, 1996a, 1996b; Kania et al., 2003; Massonneau et al., 2001; Neumann et al., 1999, 2000; Penaloza et al., 2002; Shane et al., 2003b). This precise knowledge of secretion physiology provides an opportunity for a better understanding of the plant-bacteria interactions.

Three major cluster root growth stages have been characterized by Neumann et al. (1999): the juvenile, the mature and the senescent stages. These three stages differ in quality and quantity of organic acid secreted: at the juvenile stage, cluster roots exude mainly malate and do not acidify the soil. When cluster roots reach maturity, they secrete much higher amounts of carboxylates, mostly citrate. At this stage, a strong acidification of the soil is observed. However, carboxylate secretion and acidification are biochemically independent processes. Carboxylates are secreted in the unprotonated form through very recently identified channels (Kollmeier et al., 2001; Sasaki et al., 2004), whereas acidification is due to the activation of the plasma membrane proton pump (Yan et al., 2002). At the senescent stage, only low amounts of carboxylates are secreted. The secretion of phenolic compounds has also been reported to be enhanced in cluster roots (Neumann et al., 2000). These compounds can influence the plant nutrition by metal chelation (Jung et al., 2003; Marschner 1995; Dinkelaker et al., 1995) as well as the growth and composition of rhizosphere microorganisms. Preliminary results have shown that the secretion pattern of phenolics also changes during cluster root growth, with the highest secretion occurring in young cluster roots (Weisskopf, L., Abou-Mansour, E., Tabacchi, R. and Martinoia, E., unpublished results).

Despite the high amounts of organic carbon released in the vicinity of cluster roots, the low pH and the changing conditions (the stages only last for two or three days) probably make white lupin's rhizosphere a selective environment for root-associated bacteria. These rhizosphere bacteria may in turn have an impact on the plant, for instance by

promoting root growth through auxin production. Auxin is involved in the lateral root growth and has been shown to play an important role in cluster root formation (Gilbert et al., 2000; Neumann et al., 2000; Skene et al., 2000). Although auxin production has been found in plant pathogenic bacteria (Glickman et al., 1998), other reports (Asghar et al., 2002; Patten and Glick, 2002) clearly demonstrated that auxin producing rhizobacteria can stimulate root growth and adventitious root formation. To our knowledge, only one case of auxin producing strains isolated from the rhizosphere of a cluster rooted species (*Alnus glutinosa*) has been reported (Gutierrez Manero et al., 1996). In this study, no direct link between bacterial auxin production and cluster root growth was established. Other ways for bacteria to interact with plant growth (Glick, 1995) could be the solubilization of phosphate or the mobilization of iron through siderophore production. Phosphate and iron are the two nutrients known up to now to induce cluster root formation when they are sparingly available in the soil (Hagström et al., 2001). One case of phosphate solubilizing rhizobacteria isolated from cluster roots has been described by Wenzel et al. (1994) in the rhizosphere of Waratah (*Telopea speciosissima*). As for siderophore producing bacteria, Marschner et al. (1997) assessed the iron stress of a siderophore producing *Pseudomonas* in the rhizosphere of white lupin and barley. They showed that iron stress was slightly reduced in white lupin's rhizosphere, as compared to barley. However, to our knowledge, no study up to now has dealt with these aspects on a general scale, testing a large number of bacteria living in the rhizosphere of cluster and non cluster roots for the ability to solubilize phosphate and acquire iron. Apart from these possible ways of promoting plant growth and nutrition, bacteria could also act as organic acid consumers (Jones et al., 1996; Pate et al., 2001), thus lowering the efficiency of the cluster root phosphate solubilization mechanism.

In this study, the bacterial communities associated with the cluster roots of white lupin were characterized using cultural methods and PCR-DGGE of 16S rDNA and RT-PCR DGGE of 16S rRNA. These fingerprinting methods allow assessing the shifts bacterial communities that occur as the root grows. rDNA-based analysis enable to see the dominant populations in terms of abundance ("present populations"), while rRNA-based analysis introduces a ponderation related to activity ("active populations"): in rRNA-

based DGGE profiles, the very active populations will be visible, even if they were not very abundant, but only the very abundant "less active" populations will be visible. Over the time scale of a growing cluster root, assessing changes in active populations could be more important than dealing only with present populations. Bacterial abundance and functions like auxin production, phosphate solubilization and siderophore production were also assessed. The aim of this work was to describe the bacterial communities associated with a cluster-rooted species of agricultural importance in terms of abundance, community structure and plant growth promoting functions.

3.2.3. Material and methods

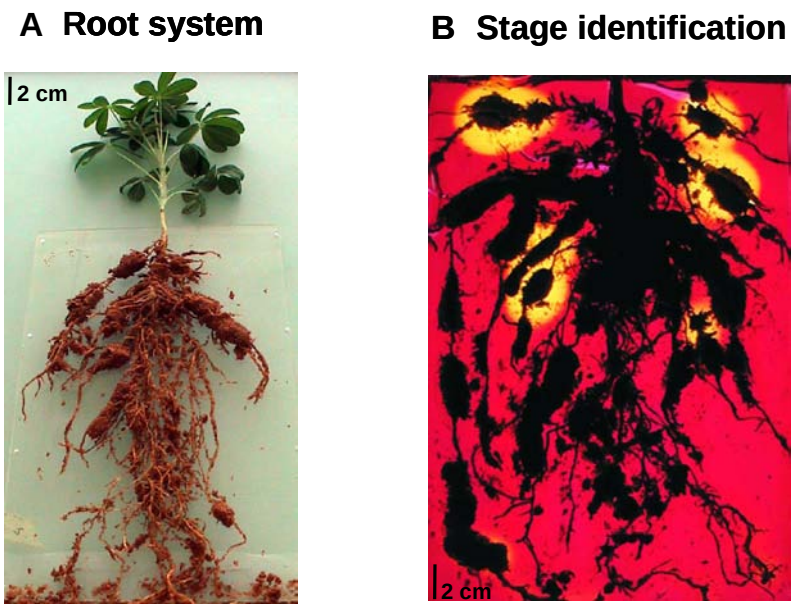
Plant material and sampling

Seeds of white lupin plants (*Lupinus albus* L. cv. Amiga, Südwestdeutsche Saatzucht, Rastatt, Germany) were incubated overnight in aerated water. After 4 days of germination in filter paper soaked in 0.2 mM $CaCl_2$, they were transferred to microcosms on 800 g of a sandy substrate. This substrate came from Nigeria and was composed of sand (91.8%), silt (7.5%) and clay (0.7%). Grain size of the sand was mostly between 0.1 and 1 mm. Prior to inoculation, the sand was tyndallised at 80 °C for two hours twice at 24 h interval. This step did not allow a complete sterilization of the sand, but reduced the original microflora by a factor of about 100. Each microcosm was inoculated with a soil (0.5 % w/w) from a field in Piemonte (Italy), where a monoculture of white lupins had been grown for over 20 years. Inoculation was performed by watering the sand with a soil suspension containing 4 g of soil in 100 ml of sterile water. Microcosms were made of Plexiglas (20 x 30 x 1 cm). pH (H_20) values were 5.7 for the sand and 4.5 for the soil used as inoculum. Phosphate content of the mixture was 0.25 mg g^{-1}. Approximatively 90 % of total phosphate was present in the inorganic form, mostly bound to iron (data not shown). To avoid iron deficiency, iron was supplied with the nutrient solution. The plants were grown at 22°C and 65% relative humidity, with a light period of 16 h at 200 µmol $m^{-2}s^{-1}$. Plants were watered twice weekly with a nutrient solution [0.05 Mm Fe(III)-EDTA, 2.5 mM $Ca(NO_3)_2$, 0.9 mM K_2SO_4, 0.8 mM $MgSO_4$, 38 µM H_3BO_3, 12.5 µM

$MnSO_4$, 1.25 µM $CuSO_4$, 1.25 µM $ZnSO_4$, 0.33 µM $(NH_4)_6Mo_7O_{24}$, 62,5 µM KCl]. Each microcosm contained a single plant and was considered as a replicate. After five weeks, four microcosms were harvested. Within each plant, different root zones were collected with adhering soils, corresponding to non cluster roots and three stages of cluster roots. Finally, a bulk soil fraction (soil devoid of root) from the same microcosm was also collected.

Stage identification and harvest

Figure 38: Harvest of the root system and identification of cluster root stages



A. Root system with adhering rhizosphere soil around cluster and non cluster roots. B. Revelation of acidification at the mature stage of cluster roots. The root system was overlaid by an agar-gel containing a pH indicator. After 20-30 min, yellow zones appeared, revealing the mature stage of the cluster roots. See material and methods for more details.

In order to characterize microorganisms from defined root zones, a crucial step was the cluster root stage identification. This had to be done as quickly as possible to prevent microbial communities from changing too much during the harvesting procedure. Plants were carefully removed from their microcosm (Figure 38 A) and placed between two layers of a 1 % agar gel containing bromocresol purple 0.01% as a pH indicator (adapted

from Marschner and Römheld, 1993 and Massonneau et al., 2001). This pH indicator is yellow up to pH 5.5, red up to pH 6.5 and purple above this value. Bromocresol purple can be found as a pH indicator in culture media at the concentration we used and should thus have no toxic effect on bacteria. After 20 to 30 minutes, stages were revealed as shown in Figure 38 B: due to the acidification occurring around mature cluster roots, the corresponding zones were characterized by yellow spots in the agar gel. Based on this phenomenon, the place of juvenile (downstream of the mature) and senescent (upstream of the mature) stages could be derived. After stage identification, the agar layer was removed and the root zones collected with their adhering rhizosphere soil (soil firmly attached to the roots). Four to seven root parts corresponding to a same root stage were pooled together. Non cluster roots and bulk soil were also collected. After washing the roots in a sodium phosphate buffer 0.1 M pH 7 (SPB) under agitation, two different fractions were obtained: the washed roots, named RE fraction (rhizoplane-endorhizosphere) and the soil remaining in the SPB, named RS fraction (rhizosphere soil). For each replicate (single plant in a microcosm), 9 samples were collected: juvenile cluster roots (RE and RS), mature cluster roots (RE and RS), senescent cluster roots (RE and RS), non cluster roots (RE and RS) and bulk soil. After washing, roots were weighed and ground in sterile SPB. A part of each root, rhizosphere and bulk soil samples was then submitted to DNA and RNA extraction while another part was used for cultural analyses.

Cultural methods

Bulk soil, RS and ground RE samples were ten-fold serially diluted in SPB and appropriate dilutions were spread in triplicates on modified Angle medium (Angle et al., 1991), which was designed to have an ionic composition similar to that found in most non-saline soils. One liter medium contained 1g glucose, 0.2 g NH_4NO_3 , 0.69 g $CaS0_4$. 2 H_2O, 0.41 g $MgCl_2$. 6 H_2O, 250 mg yeast extract, 500 µl KOH 1M, 110 µl Fe-EDTA solution (1.4 ‰ Na_2EDTA and 5 ‰ $FeS0_4$. 7 H_20 in 9 mM H_2SO_4), 68 µl KH_2PO_4 1%, 100µl of a solution containing oligoelements (0.1 ‰ $ZnSO_4$. 7 H_2O, 0.03 ‰ $MnCl_2$. 4 H_2O, 0.3 ‰ H_3BO_3, 0.2 ‰ $CoCl_2$. 6 H_2O, 0.01 ‰ $CuCl_2$. 2 H_2O, 0.02 ‰ $NiCl_2$. 6 H_2O,

0.03 ‰ Na_2MoO_4. 2 H_2O), and 20 ml Tris Buffer pH 7.0 (12 % Tris in 0.55 M HCl). pH was adjusted to 7.0. Colony forming units (CFU) were counted after five days of incubation at 24°C and calculated per gram of dry soil or roots. For isolation of strains, the replicates (same root stages coming from different plants) were pooled together in order to reach a minimal number of 30 strains per sample. A total of 30 to 80 colonies were randomly picked from appropriate dilution plates and incubated at room temperature on a standard medium (Nutrient Agar, Merck) for several days. After checking the purity of the isolates, *in vitro* tests of bacterial activities related to plant growth and rhizosphere competence were performed. The auxin production assay was performed according to Bric et al. (1991), in a slightly modified manner. Bacteria were grown for 48 h on a standard medium (Nutrient Agar, Merck) supplemented with 5 mM L-Tryptophane. Inoculated plates were overlaid by a nitrocellulose membrane (Protan BA, 45 µm pore size, Schleicher & Schuell) prior to incubation. After incubation, the membrane was removed and treated with Salkowski reagent (1.2 % $FeCl_3$ in 37 % sulfuric acid) for auxin revelation. For the siderophore production assay, the standard Nutrient Broth medium (Merck) was supplemented with a CAS solution (Schwyn and Neilands, 1987), in which iron is supplied in a complexed form with a dye, chrome-azurol. The CAS solution gives the medium a blue color. Upon siderophore production, the iron is dissociated from the dye and the medium turns orange. This test was performed in liquid culture using microplates (NUNC^{TM}), to prevent spreading of iron solubilization zones from fast-growing strains to slow-growing strains. Production of siderophores was recorded after 15 days of incubation, time after which no additional positive strain was observed. Phosphate solubilization assay was performed according to Kim et al. (1997), with a double-layer plating to enhance detection sensitivity: the lower layer contained 10 g l^{-1} glucose, 0.5 g l^{-1} yeast extract, 0.1 g l^{-1} $CaCl_2$.2 H_2O, 0.21 g l^{-1} $MgSO_4$.7 H_2O, 0.5 g l^{-1} NH_4Cl and 15 g l^{-1} agar, whereas the upper layer had the same composition except that 5 g l^{-1} $Ca_3(PO_4)_2$ were added and half of the agar concentration was used, to allow an easy and thin spreading. Phosphate solubilization was visible after one to four days by a transparent area around the colonies of phosphate solubilizing strains. Positive strains were recorded after four days. This method based on solubilization of calcium phosphate was used here despite the fact that most phosphate in

our substrate was present in a form bound to aluminium or iron. Preliminary tests using a medium containing either aluminium or iron phosphate showed a poor bacterial growth and a slow solubilization visualization, possibly due to a toxic effect of soluble Al or Fe. Thus, the calcium phosphate medium was preferred in this study, even if the mechanisms of solubilization of phosphate bound to Al or Fe *vs*. Ca might be different (Illmer and Schinner 1995).

Molecular methods

Sample storage

Approximately 0.5 g of soil and root samples were placed in FastRNA™ matrix tubes D for RNA and matrix tubes E for DNA (Bio101, QBiogene) and frozen in liquid nitrogen. They were kept at -80°C until analysis.

RNase-free conditions

All RNA handlings were performed in RNase-free conditions. Aqueous solutions were treated with 0.1 % Diethyl pyrocarbonate (DEPC). Glassware was placed at 200°C overnight and plastic material soaked in 0.1 N NaOH/1 mM EDTA solution overnight, and then rinsed with RNase-free water. RNase-AWAY solution (Promega) was used to treat the material and working areas.

DNA extraction

DNA extraction was performed using a bead beating technique (FP120 FastPrep™ cell disruptor, Savant Instruments) in combination with the FastDNA Spin Kit for Soil (Bio101, QBiogene) according to Borneman's conditions (Borneman et al., 1996), except that 500 µl of DNA lysate were extracted with 500 µl of Binding Matrix (Bio101, QBiogene). The final DNA extracts were quantified using GeneQuant (Pharmacia) and stored at -20°C before use.

RNA extraction and reverse transcription

RNA extraction and reverse transcription were performed as developed by M. Jossi (personal communication). Total RNA was extracted and purified using combination of FastRNA™ matrix D tubes (Bio101) and RNeasy® Plant Kit (Qiagen). 450 µl of RLT Buffer (Qiagen) were added to each FastRNA™ tube containing the samples. The tubes were shaken twice for 10 s at 6 ms^{-1} using a FastPrep™ cell disruptor (Borneman and Triplett, 1997). Purification was performed with the RNeasy® Plant Kit (Qiagen), according to the manufacturer's instructions. The final RNA extract was eluted in 100 µl Tris 10 mM. A DNase treatment was added in order to remove any DNA contamination: RQ1 RNase-Free DNase (Promega), corresponding buffer and Stop Reaction solution were used according to the manufacturer's protocol. The final RNA extracts were quantified using GeneQuant (Pharmacia) and stored at –80°C before use. Reverse transcription was performed using ImProm-II™ Reverse Transcription System (Promega) with random hexamer primers (Promega) and thermo-cycler model PTC-200 (MJ Research Inc.). 3.5 µl of RNA extract were mixed with 1 µl of primers (10 mM) and 0.5 µl of RNasin® Ribonuclease Inhibitor (Promega). This mixture was incubated at 70 °C for five min and chilled on ice until reverse transcription mix was added. This mix was then combined to (final concentrations) 1 x ImProm-II™ Reaction Buffer, 1 U / 20 µl RNasin, 6.0 mM $MgCl_2$, 0.5 mM of each dNTP, 5 % (vol/vol) ImProm-II™ Reverse Transcriptase and RNAse-free water in a final volume of 20 µl. The following program was used: annealing at 25 °C for five min, extension at 42 °C for one hour and inactivation of reverse transcriptase at 70 °C for 15 min. Positive and negative control reactions were performed as recommended by the manufacturer. The resulting cDNAs were stored at -20 °C.

Polymerase chain reaction (PCR)

PCR amplification of the V3 region of 16S rDNA was performed in a single step. The forward 338f (5'-ACTCCTACGGGAGGCAGCAG-3') and reverse 518r (5'-ATTACCGCGGCTGCTGG-3') universal primers (Ovreas et al. 1997) were used to

amplify the V3 region of the 16S rDNA gene. A 40 bp GC-clamp (Muyzer et al. 1993) was added on the 5' end of the forward primer for DGGE analysis. The PCR reaction mix contained (final concentrations) 1x Thermophilic DNA polymerase Buffer (Promega), 2.5 mM $MgCl_2$ (Promega), 0.025 mM of each dNTP (Gibco), 0.25 µM of each primer (Microsynth, Balgach, Switzerland) 0.01 U/µl of T4 gp32 (QBiogen) and 0.05 U/µl of Taq DNA polymerase (Promega). 0.5-1 ng/µl (final concentration) of extracted DNA and cDNA was used as template for a 50 µl reaction. The first heat denaturation step was performed at 94 °C for five min. The reaction mixtures were then subjected to 31 amplification cycles. Cycles consisted of heat denaturation at 94 °C for one min, primer annealing at 65 °C for 30 s with a touchdown of 1 °C per cycle during ten cycles (annealing temperature for remaining cycles was 55°C), and extension at 74 °C for one min. The mixture was maintained at 74 °C for 10 min for the final extension. The 220 bp PCR products were checked on a 1 % agarose gel and quantified with the Low DNA Mass Ladder (Invitrogen).

Denaturing gel gradient electrophoresis (DGGE)

DGGE was performed as developed by D. Roesti (personal communication) with a 8 % (w:v) acryl-bisacrylamide (37.5:1, Qbiogene) gel with 30-60 % linear urea/formamide (Fluka/Qbiogene) denaturing gradient (100 % denaturant corresponds to 40 % formamide + 7 M urea). 600 to 800 ng of PCR products were concentrated in a vacuum concentrator centrifuge (UNIVAPO 150H, UniEquip, Germany) and resuspended in 15 µl of water. After adding five µl of loading buffer (0.05 % bromophenol blue and 0.05 % xylene cyanol in 70 % glycerol), samples were loaded on the DGGE gel and submitted to electrophoresis in 1x TAE buffer (Qbiogene) at 60°C with a constant voltage of 150 V during five hours using a Bio-Rad D-Code Electrophoresis System (Bio-Rad). The strains used to build the reference DGGE pattern are ordered as follow after migration: *Pseudomonas fluorescens* ATCC 27663, *Bacillus subtilis* ATCC 14893, *Sinorhizobium meliloti* DSM 1981, *Flavobacterium capsulatum* DSM 30196, *Arthrobacter globiformis* DSM 20124, *Thermus filiformis* NCIMB 12588 and *Thermus thermophilus* DSM 579. The gels were stained in the dark for 20 min in 0.01 % Sybr Green I (Molecular Probes,

Leiden) in 1x TAE solution and photographed with the Multi-Analyst package from Bio-Rad (Bio-Rad). Images were normalized according to the reference patterns and the profiles were compared using the GelCompar software (Applied Maths). DGGE profiles were then converted into a numerical matrix for statistical analysis.

Statistical analyses

CFU counts were compared statistically using the Student's *t*-test ($P<0.01$). Results of *in vitro* tests of bacterial activities were validated using a χ^2-test ($P<0.05$). These analyses were performed using S-Plus 6 Statistical Software (Insightful Corporation, Seattle, WA, USA). For the analysis of the DGGE profiles, even if amplification products coming from different populations can co-migrate, each band was considered as corresponding to a single bacterial population and the band intensity (relative surface of the peak compared to the surfaces of all the peaks in the profile) as corresponding to the relative abundance of the corresponding population (Fromin et al., 2002). Bands whose average relative contribution was below 1 % were discarded. For the RE samples, bands corresponding to plastids (determined by PCR-DGGE of DNA extracted from lupin seeds germinated in sterile conditions, data not shown) were discarded prior to analysis. Richness of the profiles was revealed by the number of detectable bands and Shannon diversity index was calculated as $-\sum p_i \log_2(p_i)$ where p_i represents the relative abundance of one given population in the profile. Richness value and Shannon diversity index for a given sample were averages of three to four replicates (corresponding to different plants). Differences were statistically validated using the Student's *t*-test ($P<0.01$). The correspondence analysis was performed with three to four profiles (replicate samples coming from different plants) for each type of sample. Ordination methods were applied on the basis of the numerical DGGE data using the program *Progiciel R* (Legendre and Vaudor, 1991). The variation between the different samples based on their bacterial community DGGE profiles taking into account the relative intensity and position of the bands (Fromin et al., 2002; Marschner and Baumann, 2003) was then analyzed by correspondence analysis using the Canoco 4.0 software (Microcomputer Power, Ithaca, USA).

3.2.4. Results

Characterization of bacterial abundance associated with the different root stages

Bacterial abundance transiently decreased at the mature stage of cluster roots (Figure 39), as revealed by plate counts on the non selective Angle medium. Interestingly, this significant decrease ($P<0.01$) occurred not only in the root fraction, but also in the rhizosphere soil (going up to about half a cm distance from the root), showing that the effect of root secretion in soil is not restricted to the immediate vicinity of the roots. A similar decrease of bacterial abundance was also observed using microscopic counts after DAPI staining (data not shown), suggesting that this result is not due to a decrease in bacterial cultivability.

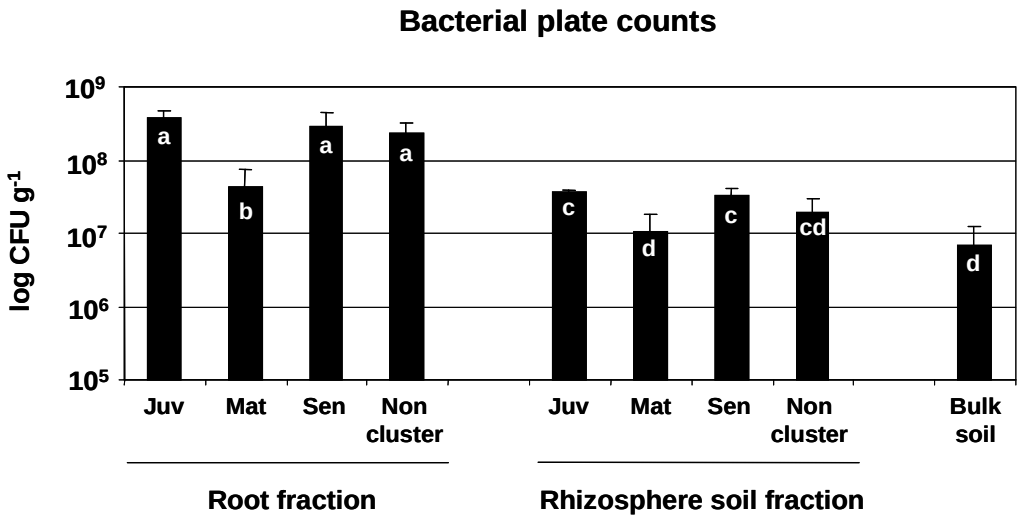


Figure 39 Numbers of CFU (colony forming units) g^{-1} dry weight in function of root stage and type, for the root fraction and the rhizosphere and bulk soil fractions. Juv: juvenile, Mat: mature, Sen: senescent. CFU were counted on the non-selective Angle medium. Values with different letters (a, b; c, d) are significantly different (Student's t-test, $P<0.01$, $n = 4$). Plate count values were only compared within a same fraction (root vs. soil).

Bacterial properties related to plant growth and rhizosphere competence

Higher frequencies of auxin producing bacteria were found to be associated with juvenile and mature roots, compared to senescent roots (Table II). However, no significant difference was observed between juvenile and mature cluster roots and non cluster roots.

No significant difference between root and rhizosphere soil fractions could be shown. Frequencies of siderophore producing strains at different stages of white lupin's cluster roots, in the root, rhizosphere soil and bulk soil fractions are also shown in Table II. A significant decrease in siderophore producers was observed in the root fraction of mature cluster roots. The frequencies of phosphate solubilizing bacteria were similar between the different stages or between root, rhizosphere soil and bulk soil fractions (Table II). Overall, these frequencies were quite high (more than 50% of the tested strains).

Table II

Table II Frequencies (in % of total isolated bacteria) of bacteri a producing auxin, siderophores and solubilizing phosphate in function of root age, type and proximity. About 300 randomly picked isolates were tested, at least 30 per sample. Isolated strains coming from different plants were p ooled to reach a representative number of isolates for each root type and age. Values with different letters (a, b) are significantly different (χ^2, P<0.05). Auxin production was assessed with Salkowski reagent after growth on a standard medium supplemented with L-tryptophane. Siderophore production was visualized by color change after growth on a standard culture medium supplemented with CAS solution. Phosphate solubilization was determined on a medium containing calcium phosphate.

	Root fractions				Soil fractions				
	Juv	Mat	Sen	Non cluster	Juv	Mat	Sen	Non cluster	Bulk soil
Auxin	33.8 ^{a}	17.3 ^{a}	4.5 ^{b}	19.2 ^{a}	26.6 ^{a}	22.5 ^{a}	9.7 ^{b}	27.3 ^{a}	7.0 ^{b}
Siderophores	58.1 ^{a}	20.7 ^{b}	43.6 ^{ab}	70.8 ^{a}	33.3 ^{b}	34.6 ^{ab}	48.0 ^{ab}	50.0 ^{ab}	61.3 ^{a}
Phosphate	71.4 ^{a}	55.1 ^{a}	60.8 ^{a}	65.7 ^{a}	56.8 ^{a}	75.6 ^{a}	66.2 ^{a}	62.9 ^{a}	56.6 ^{a}

Bacterial community structure assessed by PCR-DGGE of 16S rDNA and 16S rRNA

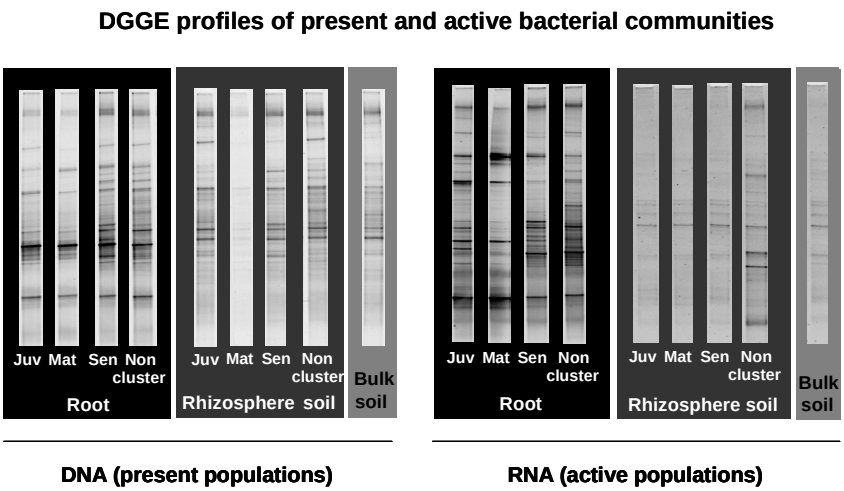
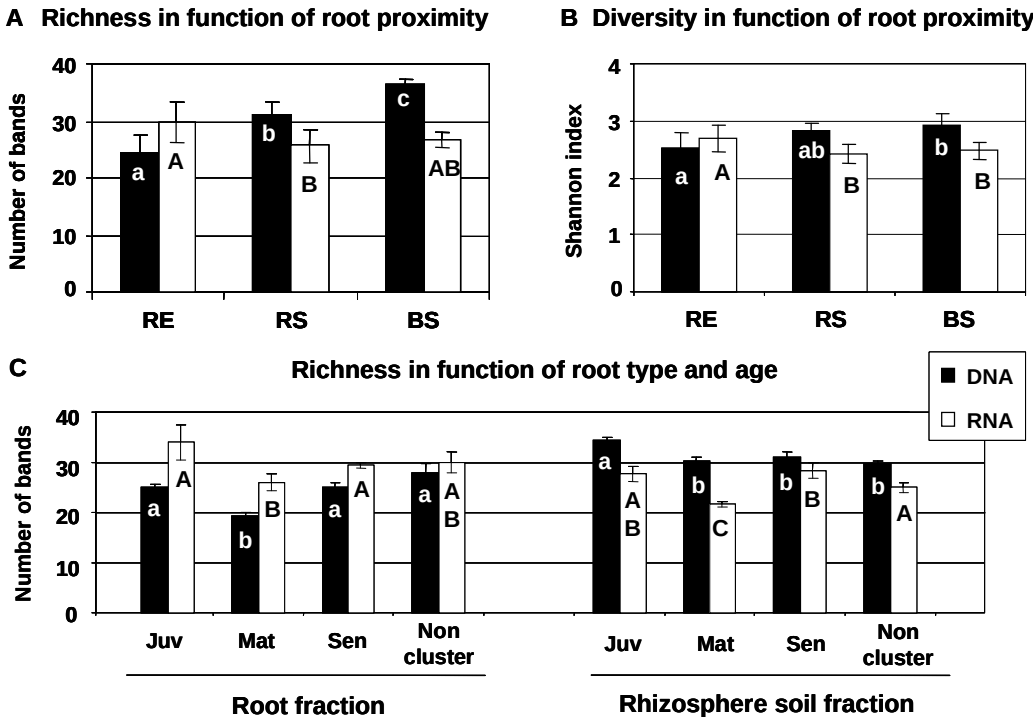


***Figure 40: Example of DGGE profiles** of the present (rDNA-based) and active (rRNA-based) bacterial communities at the different root stages, types and proximity.*

From the DGGE profiles obtained (Figure 40), two aspects related to bacterial diversity were quantified, namely the Shannon index (diversity) and the number of bands (richness). Since in almost all cases, these two parameters followed the same pattern, they are only shown in the first figures (41 A and 41 B). Only results for the richness are presented thereafter. A highly significant effect ($P<0.001$) of root proximity on sample richness and diversity (Shannon index) was observed for the DNA-based profiles.

Figure 41: Richness and diversity of the bacterial communities according to root proximity, type and age.



Richness was determined by counting the number of bands per DGGE profile and Shannon index was calculated as $-\sum p_i \log_2(p_i)$ where pi is the relative abundance of a given band in the profile. Letters (a, b, c) stand for statistically significant differences (Student's t test, $P<0.01$, $n = 3$-4). Black: DNA, white: RNA. A. Richness of bacterial communities according to root proximity. RE: root; RS: rhizosphere soil; BS: bulk soil. B. Shannon indices of bacterial communities according to root proximity. C. Richness of bacterial communities according to root stage and type.

Figures 41 A and 41 B show the selective effect of roots on the community DNA profiles: a decrease of the richness (number of bands, Figure 41 A) and of the diversity (Shannon index, Figure 41 B) from the bulk soil to the rhizosphere soil and finally to the root fraction. For the active populations (RNA profiles), the opposite tendency was observed. For active communities, there was a significantly higher diversity of active populations in RE than in RS fraction, which can be related to the stimulating impact of the direct root vicinity on the microflora. Figure 41 C shows the richness of bacterial communities in function of root type and cluster root stage: a significant decrease in richness was observed in mature cluster roots, both in RE and RS fractions, indicating a strong selection of populations at this stage of high organic acid and proton secretion. This decrease was noticed both for the present and the active populations

In order to look for the most discriminating factors influencing the bacterial communities, DGGE profiles were analyzed by correspondence analysis (Figure 42). The result of the global correspondence analysis on all the samples is presented in Figure 42 A.

Figure 42: Correspondence analyses based on DGGE profiles

A Global correspondence analysis of the DGGE profiles

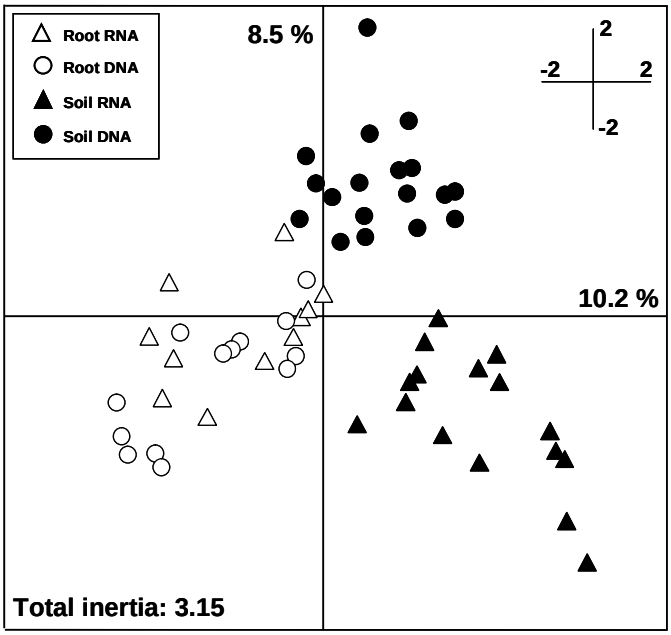
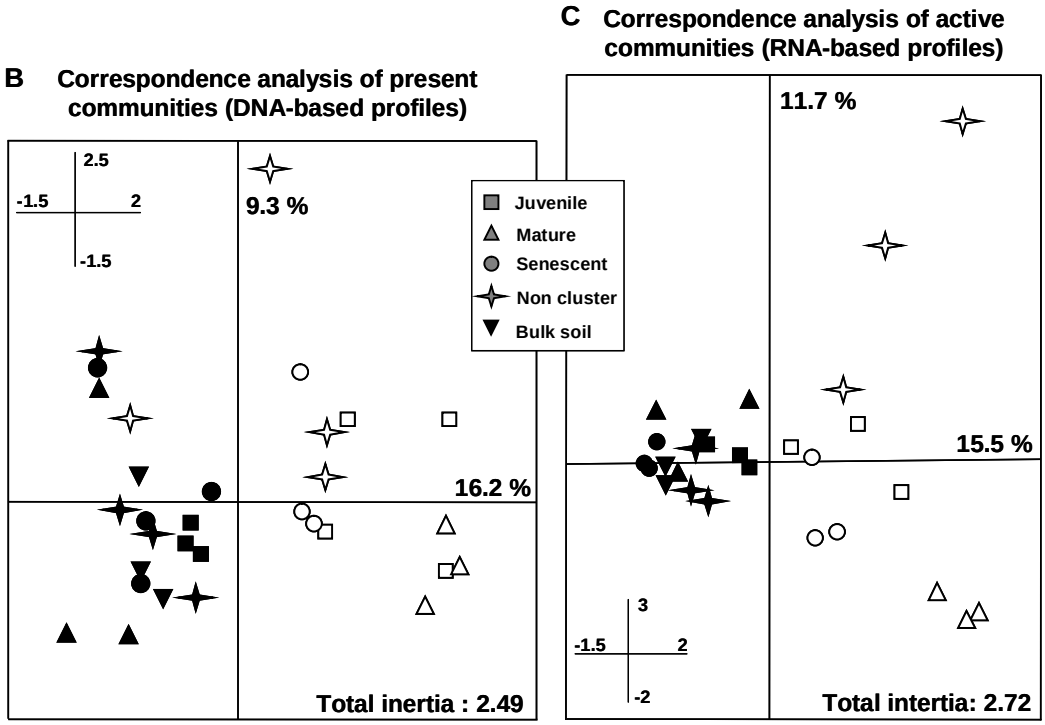


Figure 42 (cont.): Correspondence analyses based on DGGE profiles



*Correspondence analyses of bacterial community profiles for different root types, stages and proximity using 16S rDNA- and rRNA-based DGGE profiles (n = 3-4). Explanation percentage of the axes are calculated as eigenvalue*inertia^{-1}. A. Global analysis of rDNA- and rRNA-based samples. Empty symbols: root; Filled symbols: soil; circles: DNA; triangles: RNA. Eigenvalues of first and second axes are 0.32 and 0.27. B. Correspondence analysis of present bacterial communities, based on rDNA profiles. Eigenvalues of first and second axes are 0.40 and 0.23. C. Correspondence analysis of active bacterial communities, based on rRNA profiles. Eigenvalues of first and second axis are 0.42 and 0.32. For Figures 5B and 5C: empty symbols: root; filled symbols: soil; squares: juvenile; triangles: mature; circles: senescent; stars: non cluster; inverted triangles: bulk soil.*

Bacterial communities living in the root vicinity (empty symbols) were very different from those isolated from the rhizosphere and bulk soils (filled symbols). Moreover, present (circles) and active (triangles) communities were more different from each other in the soil fraction than in the root fraction. This suggests that bacterial communities living in the root and at its surface are generally active. A similar analysis was performed separately on present (DNA-based) and active (RNA-based) communities. In present communities (Figure 42 B), the cluster and non cluster roots did not group separately,

suggesting that the bacterial communities associated with the two types of roots were not very different. Moreover, no grouping between bacterial communities associated with the cluster root stages occurred in the present communities, but the variability between replicates was smaller than for non cluster roots. This could be explained by a more selective impact of cluster roots on the microflora, or by the fact that the samples collected for non cluster roots were more heterogeneous with regard to age than the samples collected for cluster roots. In the root fraction (empty symbols), the structure of active communities (Figure 42 C) was more influenced by root type and cluster root age than the structure of present communities (Figure 42 B). A further interesting point in active communities (Figure 42 C) was that the bacterial communities isolated from the root fraction of the mature samples (empty triangles) were well separated from all other samples, and especially from the non cluster roots. This could be related to the strong selective impact of root exudation of organic acids and protons on associated bacterial communities.

3.2.5. Discussion

In order to better characterize the bacteria living in the rhizosphere of white lupin's cluster roots with respect to their abundance, functions and diversity, both a classical cultural approach and a molecular approach based on DNA and RNA fingerprinting were used. Each method suffers from some limitations, for instance the fact that only 1 % of bacteria are culturable (Amman et al., 1995) or that among the 16S rRNA sequences, some are more easily amplified than others (Reysenbach et al., 1992). The bias due to persistence of intact DNA remaining in soils after cell death can be reduced by using a RNA-based profiling in parallel to DNA, since RNA is very rapidly degraded in the soil conditions. The best way to overcome these methodological limitations is to use complementary approaches, in order to investigate the problem from different perspectives. Enlarging the spectrum of information allows comparison of the results obtained with different approaches.

A good example in our case was the decrease in bacterial abundance at the mature stage of cluster roots, which was observed with cultural methods, and confirmed with a direct microscopic approach. Moreover, this stage was also characterized by a decreased number of DGGE bands, reflecting a reduced richness and diversity of the bacterial communities. Both results suggest that the mature stage exerts a highly selective effect on the associated microflora. This effect, which occurs precisely at the moment of high citrate and proton exudation, could be viewed as a way of the plant to protect its phosphate solubilizing agents from being degraded by microorganisms. One can speculate that the pH plays the major role in decreasing bacterial abundance, since it is known from previous studies (Neumann et al., 2000) that the pH around the cluster roots of white lupin can be strongly decreased. A test made to assess the ability of bacterial strains isolated from different cluster root stages to grow in acidic culture media confirmed the hypothesis that low pH is probably the major factor limiting bacterial growth (data not shown). Acidification, which parallels citrate secretion in white lupin (Neumann et al. 1999, Massonneau et al. 2001), is therefore not only useful for solubilizing phosphate bound to calcium, but may also maintain bacterial populations at a lower level, hence reducing bacterial consumption of citrate and enhancing its efficiency in phosphate solubilization (Jones et al., 1996; Pate et al., 2001). It should be mentioned here that carboxylate secretion does not always induce acidification. On the one hand, there are cases where the secretion of carboxylates is not accompanied by acidification of the rhizosphere, for instance young cluster roots of white lupin (Neumann et al. 1999). On the other hand, acidification may occur without changes in organic acid secretion, as reported for rape (Hedley et al., 1982). For more details on acidification and organic acids in the rhizosphere, see also Jones (1998).

To evaluate the potential plant growth promoting functions of the rhizobacteria associated with white lupin's cluster roots, three physiological tests of particular interest for us were performed: siderophore production, phosphate solubilization and auxin synthesis. It should be kept in mind that *in vitro* tests only give an idea of a potential activity. Such tests can thus only indicate that this activity could occur *in situ*. However, the ability to produce auxin or siderophores, as well as to solubilize phosphate *in vitro* is a prerequisite

for an activity in the rhizosphere. The tests on bacterial activities related to plant growth promotion and rhizosphere competence showed the influence of cluster roots, especially at the mature stage, on siderophore producers, as well as the influence of root age on auxin producing bacteria. Concerning iron mobilization, our results demonstrated that the proportion of siderophore producing rhizobacteria was significantly reduced in the direct vicinity of mature cluster roots. Since in our system, phosphate was mainly available in a Fe-bound form, one can assume that the high release of citrate which solubilizes phosphate will also make iron more available to the plant and bacteria. In addition, mature cluster roots have a high reduction capacity (Dinkelaker et al., 1989; Gardner et al., 1982b). From this point of view, it is not surprising that mature cluster roots showed less siderophore producers in their vicinity: bacteria probably did not need to produce these iron solubilizing compounds, since iron is already solubilized by the plant. These results confirm previous findings (Marschner et al., 1997), which indicated that siderophore production might not be an essential feature for colonization of white lupin's cluster roots. Phosphate solubilizing bacteria were found in high frequencies (more than 50% of the tested strains) in all types of roots, but in contrast to what was observed by Wenzel et al. (1994) in waratah, we did not find any evidence for an enrichment of phosphate solubilizing bacteria in the rhizosphere of white lupin's cluster roots. The frequency of auxin producing bacteria was not higher in cluster roots than in non cluster roots. However, compared to senescent cluster roots, a higher percentage of auxin producers was found in growing roots and especially in juvenile cluster roots, where the auxin-dependent initiation of lateral rootlets occurs. It would be interesting to be able to investigate the ability of bacteria to produce auxin in root zones where cluster roots are initiated. Since auxin is needed for induction and initiation of cluster roots, bacterial supply of auxin would be most useful prior to cluster root formation and therefore, it is not surprising that the frequencies of auxin producing isolates were not higher in cluster roots than in non cluster roots.

The molecular fingerprinting technique based on DGGE analysis of the 16S rDNA and 16S rRNA fragments allowed to obtain a more general view of the bacterial communities associated with the cluster roots of white lupin. However, the fact that these

fingerprinting methods only reveal dominant populations has to be borne in mind for interpretation of the results. This means that very active populations, which are present at low densities, could be detected only in rRNA profiles, whereas other populations with low activity could be detected in rDNA, but not in rRNA profiles. Our results showed that both cluster and non cluster roots have the same two-sided effect on bacterial communities: a reduced diversity (Shannon index and richness) and an enhanced proportion of active populations among the present populations (Figures 41 A and 41 B). The latter was also demonstrated by the higher similarity between DNA and RNA profiles for communities coming from the RE fractions, compared to the RS and BS fractions (Figure 42 A). One can assume that this enhanced proportion of active bacterial populations in the proximity of the root is mainly due to the root secretions. Overall, the richness of bacterial communities associated with juvenile and senescent cluster roots did not vary significantly from these associated with non cluster roots, but a highly significant decrease in richness characterized the mature stage of cluster roots (Figure 41 C). This confirms the hypothesis that mature cluster roots have a strong selective impact on the associated microflora, probably due to the low pH and enhanced organic acid secretion. These observations, on the one hand, stress the relevance of studying separately the different stages of white lupin's cluster roots (Neumann et al., 1999; Massonneau et al., 2001) and on the other hand, show the ability of the bacterial communities to react very rapidly to changing environmental conditions. The effect of root type and age on the structure of bacterial communities living in the root vicinity was clearer in active communities (Figure 42 C) than in present communities (Figure 42 B). Thus, in our study, monitoring changes in active communities proved to be more informative than dealing only with present communities, even if in the case of Marschner et al. (2002), significant differences between the different root stages could be shown using DNA-based analyses. Several factors could explain that both studies did not come to the same conclusions: (i) the plant growth conditions (sand used, inoculation procedure and phosphorus supply), (ii) the separation of RE and RS fraction in our case, which allowed to investigate the influence of root stage at different levels of root proximity, and (iii) the data analysis (correspondence analysis *vs.* canonical correspondence analysis). But in our opinion, the most important difference was the harvest procedure: Marschner et al. (2002)

collected roots, which grew directly on the upper lid of the microcosm. The advantage of this method is that it allows a collection of root exudates and afterwards an interesting correlation of these data with the microbial community profiles, but the disadvantage is that the root samples analyzed are not surrounded by soil and hence the contact with the microflora is reduced compared to roots growing inside the microcosm. Consequently, it is not surprising that in the results obtained by Marschner et al (2002), root exudates play a more pronounced role than in our experiment, since the influence of the soil is reduced compared to the influence of the root. With our stage identification method, we are able to visualize the acidification, which, combined with the detailed knowledge acquired on cluster root exudation, allowed us to collect well-defined root samples surrounded by rhizosphere soil. This method was used here for the first time with soil still adhering to the roots and showed the strong power of acidification of the mature stage of cluster roots in white lupin. Moreover, this method is little invasive and reveals the different stages of cluster roots within 20 to 30 minutes. This is an important advantage when considering the changes that occur rapidly in the microbial communities during the sample processing.

Even if there remain many gaps in our knowledge of the rhizosphere microflora associated with the cluster roots of white lupin, this study demonstrated that using root systems with well-defined and spatially separated metabolic processes, as in the case of white lupin, allows to follow the dynamics of microbial communities and to draw conclusions concerning the metabolic activity of a root and the community structure and function of the associated microflora.

3.3. ASSOCIATIVE NITROGEN-FIXING BACTERIA IN THE RHIZOSPHERE OF WHITE LUPIN'S CLUSTER ROOTS

3.3.1. Abstract

The diversity of associative nitrogen-fixing bacteria in white lupin's rhizosphere was assessed using the dinitrogene-reductase gene (*nifH*) as a molecular marker. *NifH* was amplified from both DNA and RNA (cDNA) from different root types and root proximities. We applied RFLP analysis and a cloning-sequencing approach to compare the nitrogen-fixing communities associated with cluster roots and non cluster roots, as well as with different cluster root developmental stages. We could amplify the *nifH* gene from the extracted DNA of all root samples except from mature cluster roots. For the amplification from the cDNA, positive signals were observed for all replicates of non cluster roots, as well as in juvenile and senescent cluster roots in one out of four replicates. This suggests that associative nitrogen fixation might take place in cluster roots, in addition to the symbiotic nitrogen fixation occurring in nodules usually formed in non cluster roots. The RFLP analysis revealed differences between the patterns of nitrogen-fixers associated with cluster roots and non cluster roots (40-50 % similarity) and in the patterns associated with different stages (juvenile or senescent) of cluster roots (40-70 % similarity). The sequences retrieved from cluster roots shared a high similarity with known *nifH* sequences affiliated to *Bradyrhizobium* sp., whereas sequences coming from non cluster roots were more distantly related to *Bradyrhizobiae* and more closely to *Vibrio* or *Methylococcus* species.

3.3.2. Introduction

White lupin is an annual, leguminous plant which does not form any mycorrhizal association but is still able to grow in soils with low available phosphate thanks to the formation of cluster roots (Dinkelaker et al., 1995; Lamont, 2003; Neumann and Martinoia, 2002; Purnell, 1960). These cluster roots exude large amounts of organic molecules, mainly organic acids like citrate and malate, into the rhizosphere, as well as protons and phenolic compounds (Dakora and Phillips, 2002; Dinkelaker et al., 1997; Neumann et al., 2000). White lupin has become a favorite model plant to investigate cluster root related processes and many studies have been conducted to better understand this very efficient P acquisition strategy (Gardner et al., 1982b, 1982c, 1983; Johnson et al., 1994, 1996a, 1996b; Kania et al., 2003; Neumann et al., 1999, 2000; Penaloza et al., 2002; Shane et al., 2003b). Four major cluster root stages have been defined in white lupin (Massonneau et al., 2001): juvenile cluster roots are still growing and secrete little amounts of malate, while immature cluster roots are fully expanded and secrete low amounts of citrate and malate. Few days later, cluster roots reach the mature stage, where most of the citrate secretion occurs and a concomitant release of protons causes rhizosphere acidification. Finally, senescent cluster roots do not secrete organic acids anymore and the pH in their environment increases. The rhizosphere of cluster roots represents an environment, where organic carbon availability, pH and redox potential vary in a drastic and very fast manner, parameters which have been shown to influence the bacterial communities living in the vicinity of white lupin's cluster roots (Marschner et al., 2002; Weisskopf et al., 2005).

In addition to phosphate nutrition, white lupin is also able to acquire nitrogen through association with symbiotic nitrogen fixing bacteria belonging to the genus *Bradyrhizobium* (González-Sama et al., 2004; Robinson et al., 2000), to the genus *Mesorhizobium* (González-Sama et al., 2004) and, as very recently discovered, to the genus *Ochrobactrum* (Trujillo et al., 2005). The possibility to acquire both nitrogen through symbiotic fixation and phosphorus through cluster root formation makes white lupin a very useful crop for low input agriculture. Studies on nitrogen fixation in white

lupin have been focused mostly on nodules and symbiotic fixation (González-Sama et al., 2004; Guasch et al., 2001; Lucas Garcia et al., 2004; Robinson et al., 2000; Trujillo et al., 2005) and they were to our knowledge always performed in P-sufficient conditions. Therefore, nitrogen fixation and cluster root physiology have seldom been investigated in a same study, except by Sas et al. (2002), who analyzed the effect of nitrogen nutrition (NH_4, NO_3 or N_2) on the production and proton extrusion of cluster roots. They found that the increase in the number of cluster roots induced by P deficiency and the proton extrusion were lower for nitrogen-fixing lupins than for lupins supplied with $(NH_4)_2SO_4$, KNO_3 or NH_4NO_3.

Until now, nitrogen fixation in white lupin has only been investigated in nodules. In the present work, we assessed the question of associative fixation in white lupin's rhizosphere and especially in the cluster root environment. If phosphate and nitrogen are both limiting, white lupin will form both cluster roots and nodules. In most cases, nodules only form in non cluster roots and the two structures are thus spatially separated. Since bacterial nitrogen fixation is a very costly process in terms of energy and organic carbon, we postulated that the rhizosphere of cluster roots, which is particularly rich in citrate and malate, would constitute a favorable environment for associative nitrogen-fixing bacteria. Moreover, the high respiration rate of these roots (Neumann et al., 2000) is likely to lower the oxygen concentration in the root proximity, an additional favorable aspect, since the key enzyme of the nitrogen fixation, the nitrogenase, is inhibited by high levels of oxygen. Based on this physiological knowledge of cluster root physiology, we asked the question whether we would find an enrichment of associative nitrogen-fixing bacteria in the rhizosphere of cluster roots, in addition to the symbiotic fixing bacteria associated with non cluster roots. We hypothesized that the mature cluster root environment, with its very high citrate concentration and respiratory activity, might offer better conditions for nitrogen-fixing bacteria than the other cluster root stages.

In order to investigate the potential associative nitrogen-fixing bacterial communities in the rhizosphere of white lupin's cluster roots and non cluster roots, we applied a molecular approach based on the use of the *nifH* gene as a tracer of potential bacterial

nitrogen fixation. The *nifH* gene encodes the Fe protein subunit (dinitrogenase reductase) of the nitrogenase and is, as such, present in all nitrogen-fixing bacteria. Interestingly, even if nitrogen fixing bacteria are found in various taxa, which can be evolutionarily very distantly related, it has been shown (Young, 1992) that *nifH*-based phylogenic trees share important similarities with the 16S rRNA-based phylogenic tree of nitrogen fixers. As a consequence, *nifH* has been previously used to investigate the diversity of nitrogen fixers in various environments (Brown et al., 2003; Hamelin et al., 2002; Poly et al., 2001a; Ueda et al., 1995; Widmer et al., 1999)

We investigated the diversity of associative nitrogen-fixing populations by RFLP analysis of the *nifH* fragments amplified from the DNA and RNA extracted from different rhizosphere samples of white lupin. We compared *nifH* pools associated with cluster roots (juvenile, mature or senescent cluster roots) and with non cluster roots, as well as pools coming from the rhizoplane-endorhizosphere (RE) fraction and from the rhizosphere soil (RS) fraction. In addition, a cloning and sequencing approach was also used to identify nitrogen fixing populations in the different samples studied.

3.3.3. Material and methods

Plant material and sampling

Plant cultivation, harvest, stage differentiation and separation of the root and the rhizosphere soil fractions were performed as described in chapter 3.2. Plants were grown on inoculated sand and a diluted nutrient solution was used for watering. This nutrient solution contained also nitrate but despite this N fertilization, nodules could be observed in non cluster roots. Nine samples were harvested (juvenile cluster roots (CR) RE, juvenile CR RS, mature CR RE, mature CR RS, senescent CR RE, senescent CR RS, non cluster roots NCR RE, NCR RS and a bulk soil sample). Four replicates were used, *i.e.* four different plants were harvested and the corresponding root system separated into the different fractions.

DNA and RNA extraction, Reverse Transcriptase and quantification of DNA and cDNA

DNA and RNA extraction, as well as the reverse transcriptase reaction (RT) were performed following the protocol described in details in chapter 3.2. For the RT, random primers were used, after a non-successful trial to use the *nifH* specific primers. Prior to PCR amplification, DNA and cDNA were quantified with a capillary spectrophotometer (absorbance measured at 260 nm). DNA had to be 5 times concentrated (vacuum concentrator centrifuge, UNIVAPO 150H, UniEquip, Germany) to reach the appropriate concentration, while RNA was already concentrated enough. 40 ng DNA and cDNA were used for the PCR reactions.

PCR Amplification of the nifH gene

A region of 342 bp of the *nifH* gene was amplified with the degenerated primers defined by Poly et al. (2001b): PolF (TGC GAY CCS AAR GCB GAC TC) and PolR (ATS GCC ATC ATY TCR CCG GA). Y stands for C or T, S for G or C and R for A or G. The forward primer hybridizes at the 115 position and the reverse primer at the 457 position (defined with reference to the *A. vinelandii nifH* coding sequence, Genbank, accession number M20568). They have been shown to amplify the *nifH* gene from a broad spectrum of nitrogen-fixing bacteria, an interesting feature, since we do not know which bacteria may be involved in the associative fixation in white lupin's rhizosphere.

The PCR reaction mix contained (final concentrations) 1x Thermophilic DNA polymerase Buffer (Promega), 2.5 mM $MgCl_2$ (Promega), 0.025 mM of each dNTP (Gibco), 0.25 µM of each primer (Microsynth, Balgach, Switzerland) 0.01 $U.\mu l^{-1}$ of T4 gp32 (QBiogen) and 0.05 $U.\mu l^{-1}$ of Taq DNA polymerase (Promega). 2 $ng.\mu l^{-1}$ (final concentration) of extracted DNA and 5 $ng.\mu l^{-1}$ cDNA were used as template for a 20 µl reaction. The first heat denaturation step was performed at 94 °C for three min. The reaction mixtures were then subjected to 34 amplification cycles. Cycles consisted of heat denaturation at 94 °C for one min, primer annealing at 55 °C for 60 s and extension at 74 °C for one min. The mixture was maintained at 74 °C for 5 min for the final extension.

The 340 bp PCR products were checked on a 1 % agarose gel and quantified with the Low DNA Mass Ladder (Invitrogen).

Restriction fragment length polymorphism (RFLP)

RFLP analysis was based on the use of two restriction enzymes, *Mbo*I and *Mnl*I (New England Biolabs) (Poly et al., 2001b). 5 µl of the PCR products were digested with 0.2 µl of the respective enzyme, in a total volume of 10 µl. Digestions were performed overnight to achieve complete fragmentation. Five µl of loading buffer (0.05 % bromophenol blue and 0.05 % xylene cyanol in 70 % glycerol) were added to the samples prior to loading on a 5 % polyacrylamide gel (19:1) (Bio-Rad) to allow separation of the different restriction fragments. The 20 bp ladder was used as a reference. Electrophoresis was performed in 1x TAE buffer (Qbiogene) at room temperature during eight hours (four hours at 35 V and four additional hours at 50 V) in a Bio-Rad Protean II © xi Cell (Bio-Rad). The gels were stained in the dark for 20 min in 0.01 % Sybr Green I (Molecular Probes, Leiden) in 1x TAE solution and photographed with the Multi-Analyst package from Bio-Rad (Bio-Rad). Images were normalized according to the reference patterns and the profiles were compared using the GelCompar software (Applied Maths).

Cloning-sequencing

NifH PCR products were purified using the NucleoTrap® Extraction kit for Nucleic Acids (Mascherey-Nagel) and cloned into pGEM-T vector (Promega). The transformants were randomly picked and the presence of the insert was verified by a PCR using the SP6/T7 primers and sequenced (Synergene, Switzerland). Sequences were aligned with the GeneBase software (Applied Maths) and submitted to BLAST analysis. Clustering analyses were also performed (see below).

Clustering analyses

For RFLP clustering analyses, the GelCompar software (Applied Maths) was used and the similarity analysis was based on Dice UPGMA coefficient (parameters: optimization 1 %, tolerance 2 % and minimal area 0 %).

For sequence clustering analysis, the ClustalX software (Thompson et al., 1997) was used. The tree was constructed according to the neighbor-joining method with NJplot (Perrrière and Gouy, 1996). We compared our sequences (10 for juvenile RE, 10 for senescent RE, 5 for NCR RE and 7 for NCR RS) with 17 *nifH* sequences available in the NCBI database, containing *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Vibrio*, *Methylococcus*, *Azotobacter*, *Azoarcus and Azosprillum* species, as well as two sequences affiliated to non-cultured soil nitrogen-fixing bacteria.

3.3.4. Results

Amplification of nifH *genes*

Table III shows the results for the amplification of the 342 bp fragment of the *nifH* gene obtained in the different samples from the extracted DNA and RNA. Surprisingly, no amplification was obtained from mature cluster root and rhizosphere soil samples, which indicates that despite the high exudation of carbon in form of organic anions, associative nitrogen fixation was not taking place in the rhizosphere of mature cluster roots, at least not at a detectable level. Amplification was positive for all other root samples when using the DNA as template. However, we could only reproducibly amplify cDNA corresponding to *nifH* mRNAs in the case of non cluster roots. In the rhizosphere soil fractions, amplification from the extracted DNA was possible for non cluster roots and, in two out of four cases, for juvenile cluster roots. No amplification of *nifH* from the cDNA from rhizosphere soil samples was obtained. In the bulk soil samples, no amplification was observed either in the DNA- or in the cDNA-based amplification.

Table III:* nifH *PCR amplification from DNA and RNA extracted from different samples in white lupin's rhizosphere.

RE: rhizoplane-endorhizosphere; RS: rhizosphere soil; BS: bulk soil. Numbers indicate the number of replicates (max: 4) which showed a clear positive amplification product of the expected size.

	Juvenile		**Mature**		**Senescent**		**Non cluster**		**Bulk soil**
	RE	**RS**	**RE**	**RS**	**RE**	**RS**	**RE**	**RS**	**BS**
DNA	4	2	0	0	4	0	4	4	0
RNA	1	0	0	0	1	0	4	0	0

RFLP analysis

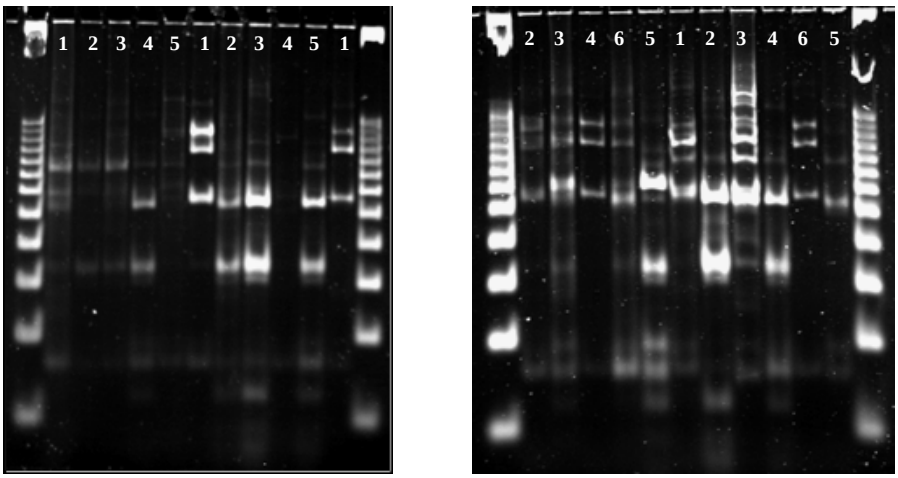
The amplified *nifH* fragments were then submitted to digestion with either *Mbo*I or *Mnl*I (New England BioLabs) in order to characterize the communities of nitrogen fixers in different samples of white lupin rhizosphere. RFLP gels after digestion with *Mbo*I (A) and *Mnl*I (B) are shown in Figure 43. Clustering analyses were performed based on these restriction patterns and several comparisons were performed: i) comparison of the communities of "potential nitrogen fixers" (DNA-based patterns) and "actual nitrogen fixers" (cDNA-based patterns) (Figure 44), ii) comparison of communities of potential nitrogen fixers in the root and rhizosphere soil fractions of white lupin (Figure 45) and iii) comparison of communities of potential nitrogen fixers associated with different root types (Figure 46).

The cDNA based profiles were characterized by a lower number of bands than the DNA-based ones (Figure 44 A and B). As expected, the bands present in the cDNA-based profiles, reflecting the diversity of the "actual nitrogen fixing bacteria" were also present in the DNA-based profiles, in the "potential nitrogen fixing bacteria", but some bands were only present in the DNA-based profiles. The 100 % similarity coefficient between replicates in Figure 44 A, as well as in Figure 45 A, is due to the fact that the analysis is

more influenced by the number of bands than by their position, where a quite large tolerance (2%, see Material and Methods) is allowed in the clustering analysis.

RFLP gels based on digestion with MboI and Mn II

A Mbo I



B Mnl I

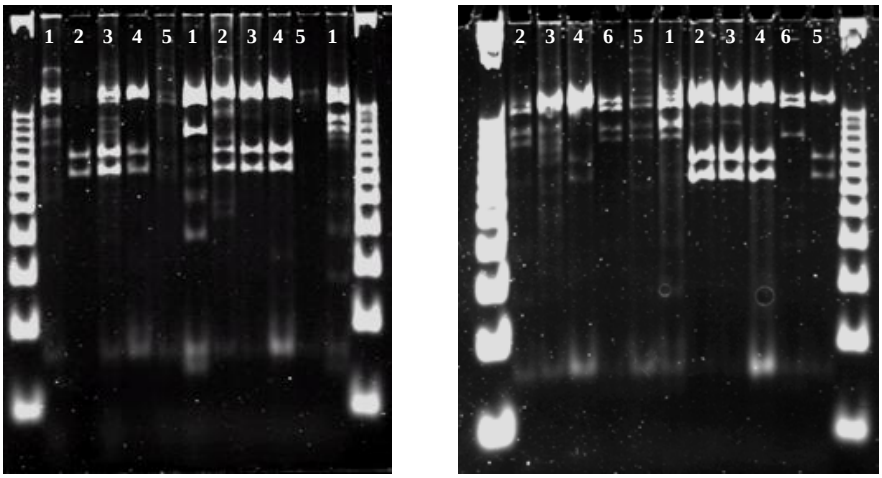


Figure 43: RFLP of* nifH *genes after digestion by Mbo I (A) and Mnl I (B).

20bp ladder was used as a reference. 1: Juvenile RE (rhizoplane-endorhizosphere), DNA; 2: Senescent RE, DNA; 3: Non cluster RE, DNA; 4: Non cluster RE, RNA; 5: Non cluster RS, DNA; 6: Juvenile RS, DNA. 4 replicates are shown, except for juvenile RS samples (6), where only two replicates showed a positive amplification.

Clustering analysis of *nifH* populations from non cluster roots amplified from DNA and RNA

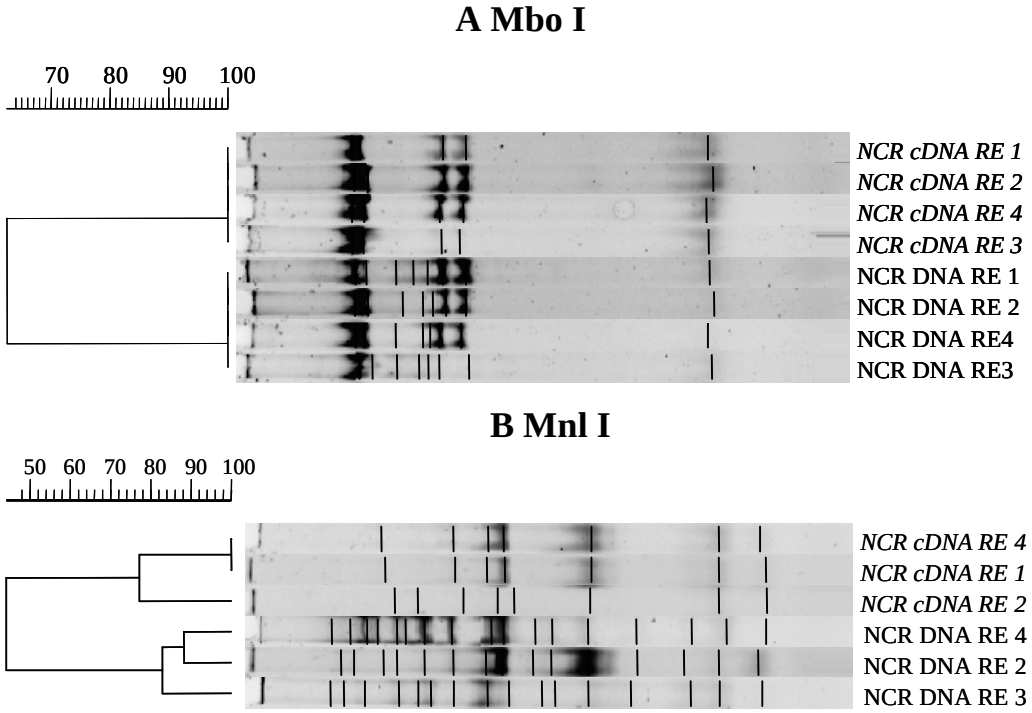


Figure 44: Clustering analyses of the RFLP profiles of* nifH *genes amplified from DNA and RNA, after digestion with MboI (A) and MnlI (B).

The scale represents the similarity between the profiles (in percentages). NCR: non cluster roots; RE: rhizoplane-endorhizosphere. The analysis was based on four replicates for MboI and three for MnlI.

When comparing root (RE) and rhizosphere soil (RS) fractions (Figure 45), it appeared that the communities of nitrogen fixing bacteria were more similar to each other within the same root type (juvenile or NCR) than within a same fraction (RE or RS), especially for the restriction with *Mnl*I (B). In the case of restriction by *Mbo*I (A), the root fraction samples of juvenile cluster roots formed a distinct group from the other samples, which is probably due to the higher number of bands present in these profiles. This higher number of bands can be related to a more frequent occurrence of the *Mbo*I restriction site along a particular *nifH* sequence present in the juvenile cluster root rhizosphere. Figure 45 B clearly indicates that there are differences (only 45 % of similarity) in the nitrogen-fixing

bacterial communities associated with either cluster roots (juvenile stage in this case) or non cluster roots (NCR) and that these differences can be observed both in the root and rhizosphere soil fractions.

Clustering analysis of DNA-based *nifH* populations from the soil (RS) and the root (RE)

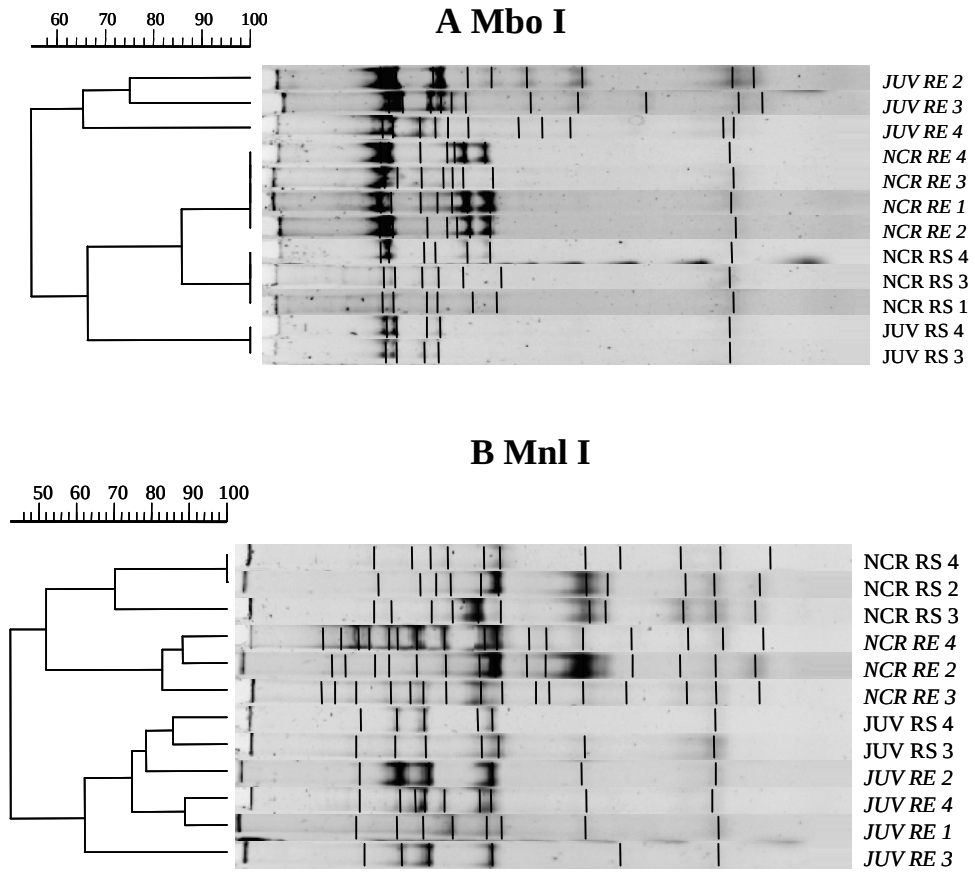


Figure 45: Clustering analyses of the RFLP profiles of* nifH *genes amplified from DNA after digestion with MboI (A) and MnlI (B) for root samples (RE) and rhizosphere soil samples (RS).

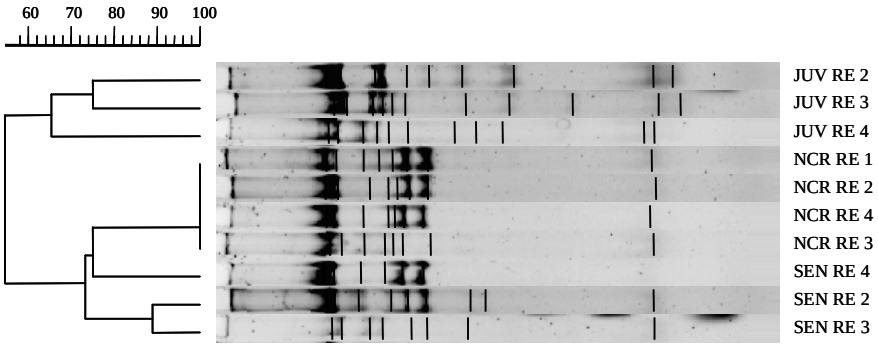
Juv: juveniles; Sen: senescent; NCR: non cluster roots; RE: rhizoplane-endorhizosphere; RS: rhizosphere soil. 2-4 replicates were used for the analysis.

The comparison of nitrogen-fixing communities associated with different root types (juvenile cluster roots, senescent cluster roots and non cluster roots) is shown in Figure 46. While the RFLP profiles of the juvenile cluster roots formed a separate cluster with

the *Mbo*I digestion (A), (like it was previously observed, see Figure 45 A), *Mnl*I profiles (B) clearly discriminated between the cluster and non cluster roots. This clustering analysis showed that nitrogen-fixing communities are influenced by the root type (cluster *vs.* non cluster), as well as by the root stage (juvenile *vs.* senescent).

Clustering analysis of DNA-based *nifH* populations from different root fractions

A Mbo I



B Mnl I

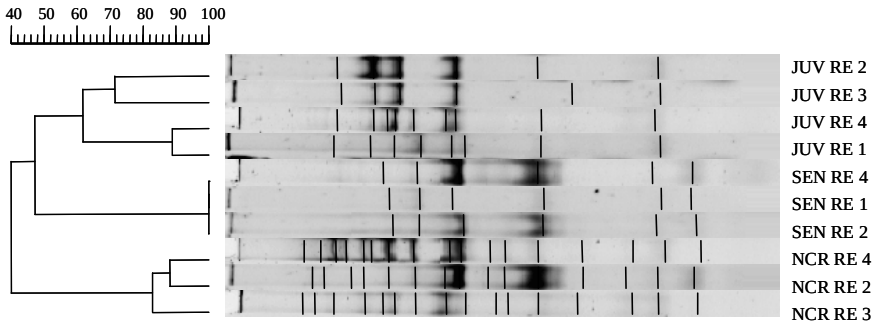


Figure 46: Clustering analyses of the RFLP profiles of* nifH *genes amplified from DNA after digestion with MboI (A) and MnlI (B) for different root samples (RE).

Juv: juveniles; Sen: senescent; NCR: non cluster roots. 3-4 replicates were used for the analysis.

Phylogenic analysis of the communities of nitrogen fixing bacteria in white lupin rhizosphere

In order to determine the phylogenetic identity of the major populations of nitrogen-fixing bacteria in the rhizosphere of white lupin, we cloned DNA-based amplification products of *nifH* fragments and sequenced the 7 clones obtained from the rhizosphere soil of non cluster roots and 26 randomly picked clones of the root fraction of non cluster roots (6), senescent cluster roots (10) and juvenile cluster roots (10). Unfortunately, we were unable to clone the *nifH* PCR products obtained from cDNAs.

Figure 47: Phylogenic tree of *nifH* sequences from cluster and non cluster roots

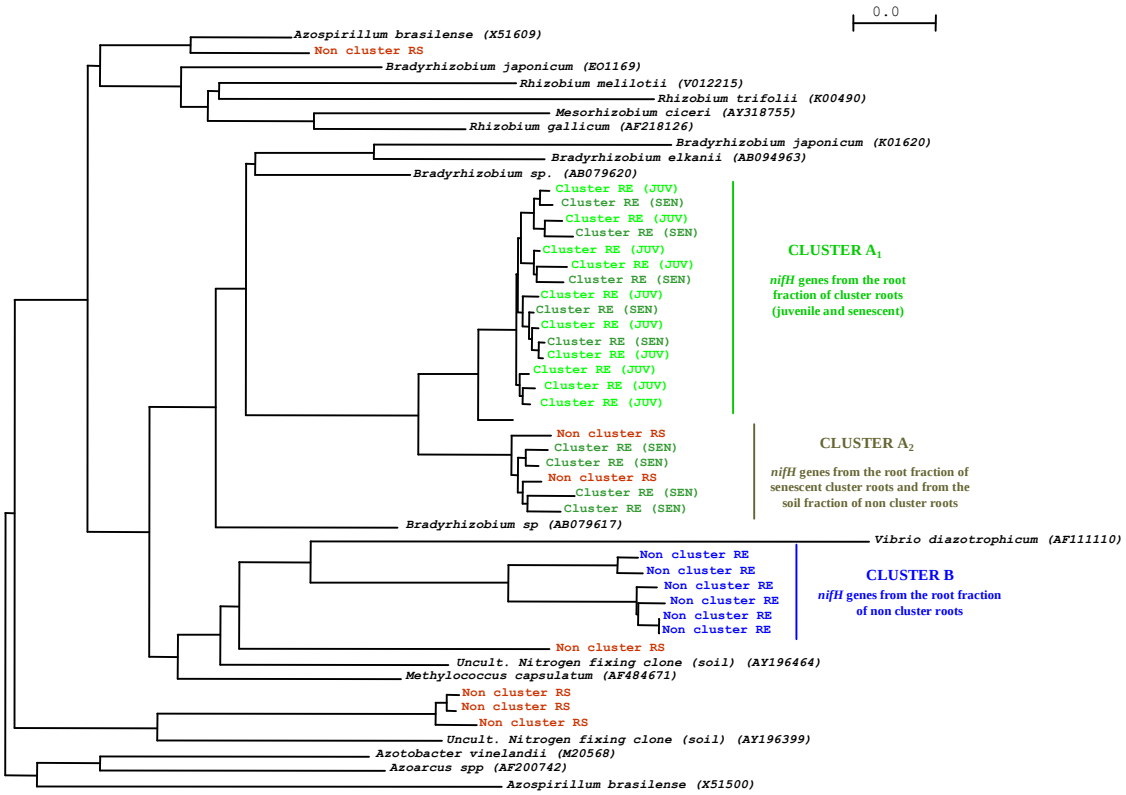


Figure 47: Phylogenetic analysis of* nifH *genes amplified and sequenced from DNA extracted from root (RE) and rhizosphere soil (RS) of white lupin.

Known sequences obtained from databases are also shown. JUV: juveniles; SEN: senescent, RS: rhizosphere soil; RE: rhizoplane-endorhizosphere. The tree was based on the alignment (ClustalX) of 342 bp of the DNA-amplified nifH gene fragment and constructed by the neighbour-joining method with NJplot. The scale bar indicates 0.02 % of sequences divergence. The percentage of 1000 bootstraps that supported each node is shown. Values below 50 % are not shown.

The results of the phylogenetic analysis of the DNA sequence of the *nifH* gene fragments are shown in Figure 47: *nifH* gene fragments isolated from the root fraction of non cluster roots were well separated from the others (Cluster B), while the sequences coming from the rhizosphere soil of the non cluster roots were distributed throughout the phylogenetic tree, indicating a higher diversity. *NifH* sequences retrieved from the communities living in the root fraction of cluster roots could be grouped in two clusters: cluster A_1, containing all the sequences coming from juvenile cluster roots, as well as some sequences coming from senescent cluster roots and cluster A_2, which contained sequences from senescent cluster roots and two sequences retrieved from the rhizosphere soil of non cluster roots. Nitrogen-fixing bacteria from the rhizosphere soil fraction were the most largely distributed within the phylogenetic tree.

3.3.5. Discussion

White lupin is a very well-studied model plant for its ability to cope with phosphate deficiency. By producing cluster roots, which secrete high amounts of citrate and malate, as well as protons, white lupin is able to acquire phosphate from pools which are not available to other plants (Braum et al., 1995). Furthermore, white lupin, as a leguminous plant, is also able to acquire nitrogen through association with symbiotic nitrogen-fixing bacteria. In addition, associative fixation through rhizosphere N_2-fixing bacteria may also take place in the vicinity of white lupin's roots. The rhizosphere of cluster roots is characterized by the availability of high amounts of organic compounds, which may supply the carbon and energy that are necessary for associative heterotrophic nitrogen-fixing bacteria. Moreover, due to the high cluster root respiratory activity, the levels of oxygen, which at high concentrations inhibits the nitrogenase activity, are likely to be reduced in such an environment. Altogether, the rhizosphere of cluster roots appears to offer the kind of conditions which are thought to be favorable for nitrogen fixation and since to our knowledge, no report exists on the associative N_2-fixation in white lupin's cluster roots, or in any cluster-rooted species, we decided to address the question in this study. We used 340 bp of the *nifH* gene as a molecular marker to analyze the diversity of

potential nitrogen-fixing bacterial communities by a PCR-RFLP approach on the one hand and a cloning-sequencing approach on the other hand.

Amplification of the nifH gene

The first surprising result was the absence of *nifH* amplification products in all mature cluster root samples, even in the rhizoplane-endorhizosphere fraction, where all other root types gave positive results. Obviously, despite the potentially favorable environment of mature cluster roots in terms of organic carbon availability and low oxygen levels, associative nitrogen-fixation is not taking place in white lupin's mature cluster roots, or at least not to a detectable level. A possible explanation for this observation is the low pH which characterizes the rhizosphere of mature cluster roots, since acidic conditions are known to decrease the survival, growth and nitrogen fixation of rhizobiae (Cheng et al., 2004; Del Papa et al., 2003; Lie, 1981). Even if *Bradyrhizobiae*, and interestingly especially the *B. lupini* species, have been reported to be acid tolerant (Macció et al., 2002; Raza et al., 2001), the experiments were usually conducted within a pH range of 4 to 10, whereas the mature cluster root rhizosphere can reach pH values below 4. Moreover, associative fixation may involve many different bacterial species and it may well be that not all are as acid tolerant as *Bradyrhizobiae*. We have shown in a previous study (Weisskopf et al., 2005) that the bacterial abundance associated with white lupin's cluster roots was significantly reduced at the mature stage and we postulated that it might be due to the dramatic and sudden pH decrease happening at this stage of root development. In this perspective, it is not surprising that in an environment where bacteria are generally inhibited, the nitrogen-fixing bacteria are not stimulated, but on the contrary, also inhibited by the low pH.

Apart from mature cluster roots, a successful amplification of the *nifH* gene was obtained for all other root samples at the DNA level and for some of them at the mRNA level, suggesting that nitrogen fixation may occur in the different root parts of white lupin in an associative manner, in addition to the symbiotic fixation occurring in nodules.

The amplification of *nifH* from soil fractions was much more limited: no positive signal at all was obtained from bulk soil samples, which is not surprising considering that we used inoculated sand and not a "real" soil and that in the absence of a root, i) the organic compounds supplying carbon and energy for the highly demanding nitrogen fixation would be lacking and ii) the soil nitrogen pool would not be as depleted as it would be in the rhizosphere due to the uptake of ammonium or nitrate by plants. In contrast, in the rhizosphere soil, *nifH* could be amplified in all four non cluster root samples, as well as in two of the four juvenile cluster root samples. However, this amplification only occurred when the extracted DNA was used as template and not with the cDNA, indicating that bacteria living in the rhizosphere soil might well have the genetic ability to fix nitrogen but would not actually express the gene and perform nitrogen fixation. Again, this could be related to the lower amounts of exudates available at a higher distance from the root surface or, since no amplification of the *nifH* cDNA could be achieved in any soil sample, it could also be due to a technical limitation, either in the extraction procedure or in the inhibition of the reverse-transcriptase or DNA polymerase by some soil components present in the insufficiently purified DNA and RNA extracts. It would have been useful here to use a constitutively expressed functional gene as a positive control for the RT-PCR analyses. In addition to this potential technical limitation, the fact that the plants were not suffering nitrogen deficiency clearly could explain this lack of selection for nitrogen fixers in the rhizosphere soil.

*RFLP analysis with MboI and Mnl*I

The RFLP analysis was performed with two different restriction enzymes, *Mbo*I and *Mnl*I (Poly et al., 2001b). Based on the percentages of similarity in the clustering analyses after restriction with *Mbo*I or *Mnl*I, it appeared that *Mnl*I had a higher discrimination power than *Mbo*I. In contrast, *Mbo*I allowed us better than *Mnl*I to see differences between juvenile cluster roots and all other samples, in the root fraction.

When comparing the potential nitrogen fixers with the actual nitrogen fixers (DNA- *vs.* cDNA-based restriction patterns, Figure 44), we observed that all fragments present in the actual nitrogen-fixing patterns were also visible on the potential nitrogen-fixing patterns, but the reciprocal was not true: a lower number of fragments could be observed in the profiles of actual nitrogen-fixing communities, reflecting the fact that possessing a gene does not mean that it is expressed and that the corresponding activity is present. This underlines the interest and importance to work on the mRNA level, even if, as mentioned above, the extraction, reverse-transcription and amplification can be delicate, especially in soil samples.

Interestingly, soil and root restriction profiles of potential nitrogen fixers were more similar within a same root type, as within a same rhizosphere fraction (RE *vs.* RS) (Figure 45). This can be related to the root selective and elective impact on the soil bacterial communities, which extends to the rhizosphere soil, as demonstrated by the presented results. The fact that no *nifH* amplification could be obtained from the rhizosphere soil of senescent cluster roots left us only the possibility to compare between juvenile cluster roots and non cluster roots, when considering also rhizosphere soil samples. If it had been possible to amplify *nifH* gene fragments from the rhizosphere soil of senescent cluster roots, it would have been very interesting to include the root and soil samples from senescent cluster roots into the clustering analysis presented in Figure 45. We could thus have seen if bacterial communities would also in the case of different cluster root stages have been more similar to each other within a same root type (juvenile or senescent) than within the same fraction (RE or RS).

The comparison of the restriction patterns of potential nitrogen fixers in the root fractions of non cluster roots, juvenile and senescent cluster roots showed that they were influenced both by the root type and the root stage (Figure 46). Even if the clustering hierarchy differed between the two restriction enzymes, the percentages of similarity between juvenile and senescent cluster root-associated potential nitrogen fixers were in both cases quite low. Cluster root- and non cluster root-associated restriction patterns also showed a low similarity percentage (below 40 %) when using the *Mnl*I enzyme (Figure

46 B), but it was not the case for *Mbo*I, where 70 % similarity was found between non cluster roots (NCR) and senescent cluster roots (Figure 46 A). Clearly, two enzymes giving such different results stress the necessity to work with many of them to obtain a clearer picture of the real diversity of the nitrogen-fixing communities, as well as of the similarities between different samples.

Phylogenetic analysis of the N_2 fixing bacterial communities

In addition to the RFLP characterization of nitrogen-fixing communities associated with white lupin's rhizosphere, we used a cloning and sequencing approach to investigate the phylogenic identity of potential nitrogen fixers in the root fraction of juvenile and senescent cluster roots, as well as in the root and rhizosphere soil fractions of non cluster roots. The bootstrap tree (Figure 47) revealed that the potential nitrogen fixers were more tightly grouped when cloned from the root than from the rhizosphere soil fraction. This is consistent with the fact that the root has a selective impact on bacterial communities and would favor specific populations of nitrogen-fixing bacteria, in the case of non cluster roots most probably symbiotic fixing bacteria. Sequences retrieved from cluster roots and non cluster roots grouped separately, indicating that the two types of roots did not harbor the same potentially nitrogen-fixing populations. This was not the case for cluster root stages, where sequences retrieved from juvenile and senescent cluster roots grouped together. Since nodules normally form on non cluster roots, one could expect that non cluster roots would attract symbiotically nitrogen fixers. It is therefore surprising that this cluster is not closely linked with known *Bradyrhizobium, Mesorhizobium or Ochrobactrum* strains, but shares more sequence similarity with a *Vibrio* or a *Methylococcus* species. In contrast, the sequences retrieved from the rhizosphere of cluster roots (both juvenile and senescent), are closely related to *Bradyrhizobiae*. This indicates that *Bradyrhizobiae* are attracted by white lupin cluster roots and that they may be involved in the associative nitrogen fixation, and not only in the symbiotic fixation. However, this has to our knowledge never been described previously. Even if some studies have reported the presence of *Rhiozobium* species in the root tissues of non legumes (Hecht-Buchholz, 1998; Kennedy et al., 2004; Rengel, 2002) and even if in these

cases, the plant growth and nutrition was improved, no evidence till now has been presented that this might be due to associative nitrogen fixation. As expected, the highest variability was found in the sequences retrieved from the rhizosphere soil samples (non cluster roots), which are well spread throughout the tree, reflecting their sequence diversity. Interestingly, our nifH sequences retrieved from soil were grouped with other sequences coming from soil (unknown nitrogen fixing clone, see Bürgman et al., 2004 for more details).

In this study, we investigated the potential nitrogen-fixing bacterial communities in the rhizosphere of white lupin cluster roots. This is, to our knowledge, the first report on a potential associative nitrogen fixation in cluster roots. *nifH* genes could be amplified in juvenile and senescent cluster roots both from the extracted DNA and, in one of four cases, from the cDNA, indicating that nitrogen fixation might also occur in the rhizosphere of cluster roots, as well as in non cluster roots. The sequence analysis revealed that these potential nitrogen fixers coming from cluster roots shared a high sequence similarity with *Bradyrhizobium* strains, suggesting that bradyrhizobiae might also be involved in associative nitrogen fixation, in addition to the symbiotic nitrogen fixation. Additional experiments where white lupin would be grown in nitrogen deficient conditions, where the nitrogen fixation activity would be assessed (through acetylene reduction) and the *nifH* mRNAs more reproducibly amplified and sequenced would give us further important results in order to conclude about the actual importance of associative nitrogen fixation in the rhizosphere of white lupin cluster roots.

3.4. BACTERIAL COMMUNITIES IN THE RHIZOSPHERE OF *ARABIDOPSIS THALIANA*: COMPARISON BETWEEN THE *TRANSPARENT TESTA 4* MUTANT AND THE WILD TYPE.

3.4.1. Abstract

The impact of phenolic secretion on the structuring of rhizosphere bacterial communities was investigated in the *Arabidopsis thaliana tt4* mutant lacking flavonoids. Phenolic contents in seeds, leaves and roots were measured in both the mutant and the wild-type grown in hydroponic cultures. The mutant was characterized by a lack of many phenolic compounds in seeds (HPLC analysis) and by the absence of flavonoids in the roots (staining with diphenyl boric acid 2 amino ethyl ester, DPBA). Present and active bacterial communities were analyzed with PCR-DGGE of the v3 region of the 16S r RNA gene. Both RNA and DNA were extracted from rhizosphere soil and roots of plants grown in pots. The richness of bacterial communities was significantly influenced by the plant genotype and a lower richness was found for present communities in the *tt4* rhizosphere. The correspondence analysis revealed that the major factors accounting for the differences in community structure were i) the root proximity (rhizosphere soil *vs.* root), ii) the type of targeted community (present *vs.* active) and iii) the plant genotype (*tt4 vs.* wt). Our results clearly show an effect of the plant genotype on the richness and structure of bacterial communities but further investigations are needed to understand if the mutant's lack in flavonoids is the major cause for these effects, or if other features of the mutant's phenotype also play a role in the structuring of bacterial communities.

3.4.2. Introduction

In order to investigate the selective and elective impact of root-secreted phenolic compounds in the structuring of rhizosphere bacterial communities, we wanted to compare the rhizosphere microflora associated with plants impaired in flavonoid biosynthesis with the corresponding non-impaired plants. Since white lupin cannot be genetically transformed and no mutants are available, we decided to study this aspect in *Arabidopsis*.

A series of mutants impaired in flavonoid biosynthesis have been described in *Arabidopsis thaliana* (Debeaujon et al., 2001; Koornneef, 1981; Shirley et al., 1992, 1995).They are called *transparent testa* (*tt*) mutants since they were identified by analysis of the seed coat. Mutants are available for all major enzymatic steps in the biosynthetic pathway of flavonoids. These mutants provide very efficient tools to investigate the physiological processes involving flavonoids and they have been extensively studied (Buer and Muday, 2004; Burbulis et al., 1996; Li et al., 1993; Peer et al., 2001). We decided to use the *tt4* mutant, which is characterized by a G to A transition in the single intron of the chalcone synthase, resulting in a null mutation in the gene (Shirley et al., 1985). Since the chalcone synthase is the very first enzyme of the flavonoid biosynthetic pathway and is encoded by a single gene in *Arabidopsis*, *tt4* should completely lack flavonoids and we expected to see more changes with such a drastic phenotype than with a less severe phenotype.

The work presented in this chapter can be divided into two parts: on the one hand, wild-type and mutant plants were cultivated in hydroponic culture to analyze the flavonoids and phenolic compounds present in seeds, leaves and roots. For the roots, in addition to the internal contents, the secretion of phenolics was also measured. An *in situ* coloration with DPBA (diphenyl-diamino-mehthylesther boric acid, Sigma) which specifically stains flavonoids, was performed on whole seedlings and root sections. On the other hand, plants were cultivated in pots on soil in order to characterize the present and active bacterial communities living in the rhizosphere soil and endorhizosphere (root tissues) of

both plants. The community structure was assessed by cluster analysis, richness calculation and multivariate analysis of DGGE profiles based on polymorphism of the v3 region of the 16S rRNA gene amplified from either the DNA samples (present populations) or the cDNA derived from the RNA samples (active populations).

3.4.3. Material and methods

Growth of Arabidopsis

The *tt4* mutant was generously provided by Prof. Winkel-Shirley (Virginia). Seed surface sterilization was performed by incubating the seeds (approx. 40 mg / Eppendorf) overnight under a fume hood in dessiccator containing 100 ml calcium hypochlorite 3 % and 3 ml of concentrated HCl in a beaker. After adding 1.5 ml of sterile agarose 0.1 %, seeds were let in the dark at 4°C for two days for vernalization. They were then transferred to either pots filled with soil (pot experiments) or to square Petri dishes containing a growth medium composed of 1.14 $g.l^{-1}$ MS (Muraashige and Skoog, 1962), 10 $g.l^{-1}$ sucrose and 9 $g.l^{-1}$ agar and incubated vertically in an illuminated growth chamber for 10 days (hydroponic culture). For the pot experiments, approximately 10 plants per pot were planted and grown for six weeks in a green house conditions in the botanical garden in Neuchâtel. The pots contained a soil collected at Eschikon, ZH (Brunisol), which was neither sterilized nor inoculated. At the harvest time, no difference in developmental stage was observed between mutant and wild-type. For the hydroponic cultures, seedlings were transferred from the Petri dishes to the hydroponic pots containing half a liter of nutrient solution (same composition as for lupin hydroponic cultures, see chapter 2.4.). Plants were grown in growth chambers (see also chapter 2.4.) for 6 weeks and nutrient solution was changed once a week.

Extraction of phenolics from seeds, leaves and roots

After 6 weeks growth, plants were harvested and separated into shoots and roots. Shoots were stored at -80 °C until further processing. Roots were weighted and incubated in 4 ml sterile water for one hour under gentle shaking. The water, containing the root exudates, was then removed, frozen and freeze-dried. The freeze-dried material was extracted with 3 ml MeOH 80 % in three successive steps, each followed by vigorous manual shaking of the tubes. After collection of the water containing the exudates, 4 ml MeOH 80 % were added to the roots and another hour of incubation was allowed to recover the internal contents of phenolics through membrane disruption. This methanol extract was then filtrated and evaporated. The phenolic fraction of leaves was obtained by grinding the leaves in 80 % MeOH, filtrating and evaporating. For the seed extraction, 20 mg of seeds were ground in 1 ml MeOH 80 % and shaken at 1400 rpm for 30 min at room temperature. Samples were then filtrated and evaporated.

HPLC analysis

Samples were resuspended in the first HPLC solvent according to the fresh weight of the original material (roots, leaves and seeds) and 50 µl were injected into the HPLC system. We used the same solvents, column, HPLC and detection devices as for the lupin analysis (see chapter 2.4.). Only the separation protocol (gradient) was different. We had to modify the protocol described by Burbulis et al. (1996), because our column was longer than the one used in this paper. The final conditions used were the following: 0.7 ml. min^{-1}, starting from 0 % B, we reached 10 % in 4 min, 15 % after 20 min, 50 % after 25 min and 100 % after 32 min. After 8 min of 100 % B, the initial conditions (100 % A) were reached again in 2 min and maintained for 8 min prior to the injection of the next sample. We determined the retention time for 10 pure flavonoids and phenolic acids known to be present in *A. thaliana* (Graham, 1991) to obtain some standards for our analyses. Compounds were detected with a UV lamp (263 nm).

In situ *staining with DPBA*

Seeds of *A. thaliana* (wild-type and *tt4*) were surface-sterilized (see above) and incubated for 2 weeks in a green chamber on ½ MS medium. For the staining, plates were prepared as follows: 5 ml of a 10 % solution of DPBA (diphenyl diaminomethylesther boric acid, SIGMA) in MeOH were added to a plate containing 100 ml of a melted suspension of agarose 1 %. The two solutions were mixed gently till the solidification occurred. Seedlings were carefully removed from the MS plates and placed onto the DPBA plates. A layer of the agarose gel containing the DPBA was placed on top of the seedling roots, to allow coloration from both sides. After 30 minutes incubation at room temperature in the dark, the upper gel layer was removed and the seedlings were submitted to UV light. A section of the roots was cut and observed with a Leica microscope under UV light.

Extraction of DNA and RNA, PCR and DGGE

For each pot (replicate), approximately 2 g of soil was collected and after washing the roots, about 100 mg could be recovered. No bulk soil sample could be harvested, since the roots had explored all the soil available in the pot, so we only discriminated between root fraction (rhizoplane and endorhizosphere) and the rhizosphere soil fraction. Each sample was separated in two fractions, one for the DNA extraction and one for the RNA extraction. These extractions, as well as the PCR, RT-PCR and the DGGE analyses, were performed according to the protocol described in chapter 3.2.

Statistical analyses

Clustering of the DNA- and RNA-based profiles was performed with the version 4.1. of the GelCompar software (Applied Maths) using Dice coefficient and UPGMA (*unweighted pair-group method using arithmetic averages*) clustering method. Multivariate analyses of the DGGE profiles were performed as described in chapter 3.2. Differences in richness were statistically validated with a Student's t test ($n = 3\text{-}4$, $P < 0.05$).

3.4.4. Results

Phenolic profiles in seeds, leaves and roots are altered in the tt4 *mutant*

For these experiments, plants were grown in hydroponic cultures. After 6 weeks of cultivation, morphological differences between the two genotypes could be observed (Figure 48). *tt4* had smaller leaves than the wild-type, with a brighter color, which may be related to the lack of anthocyanin production. No difference in developmental stage was observed.

Hydroponic cultures of *A. thaliana*

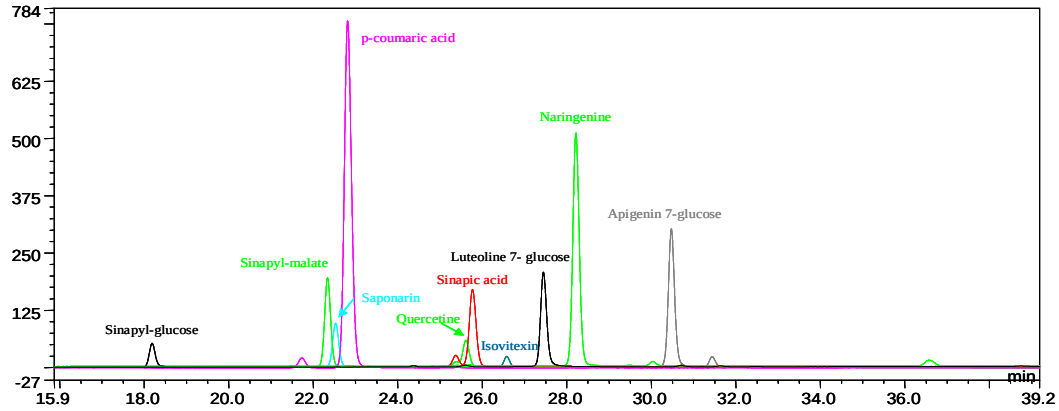
Figure 48: wt and* tt4 *in hydroponic culture

For the characterization of phenolic compounds, wild-type (wt) A. thaliana *and the* transparent testa 4 *(*tt4*) mutants were grown in hydroponic cultures for six weeks in a growth chamber. Four replicates (pots) containing 2-4 plants were used in the analysis.*

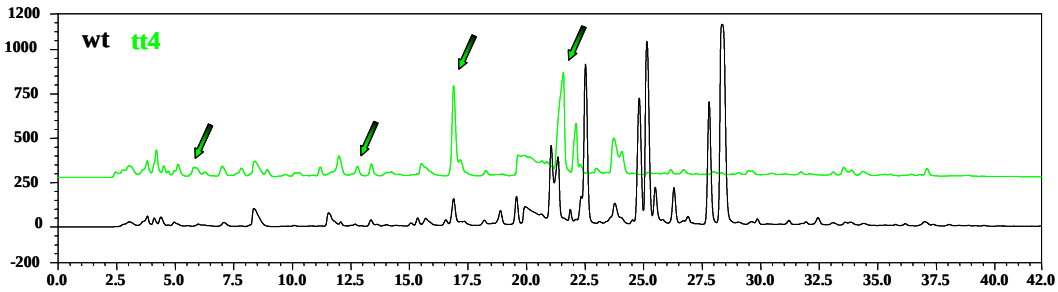
Prior to the analysis of plant samples, we adapted the HPLC separation gradient till we were able to discriminate between 10 major compounds, which we expected to be present in *A. thaliana*. We used four phenolic acids, sinapic acid, sinapyl-glucose, sinapyl-malate and p-coumaric acid, situated upstream of the chalcone synthase in the flavonoid biosynthetic pathway and which should either be not affected by the mutation or increased through accumulation. Six flavonoids were chosen among the different subfamilies: four flavones (luteoline 7-glucose, apigenin 7-glucose, saponarin and isovitexin), one flavonol (quercetin) and one flavanone (naringenin).

Figure 49: HPLC profiles of phenolic compounds of* A. thaliana *wild type and* tt4 *mutants

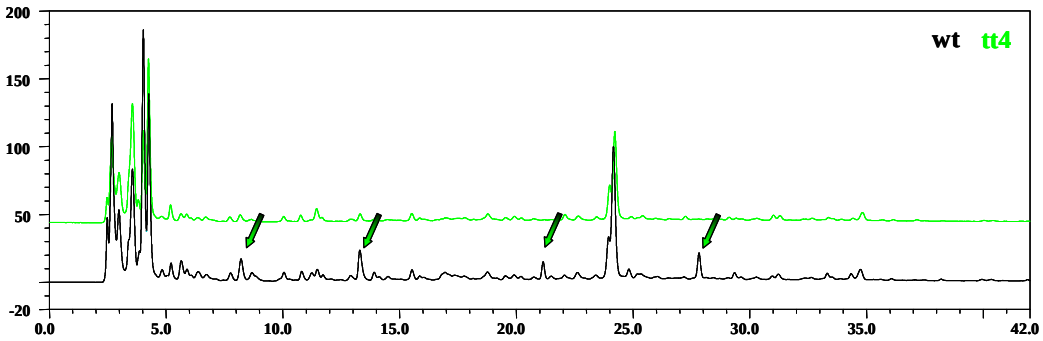
A. Separation of ten phenolic compounds used as standards



B. Seeds



C. Leaves



Analysis was performed in 40 min on a C18 reversed-phase column with an increasing acetonitrile gradient. Absorbance was measured at 263 nm. A. Separation of 10 pure compounds, used as standards. B-E. One chromatogram is shown for each sample, as a representative example of the three replicates used. B. Phenolic compounds in seeds of wt and tt4 plants. C. Phenolic compounds extracted from leaves of wt and tt4 plants.

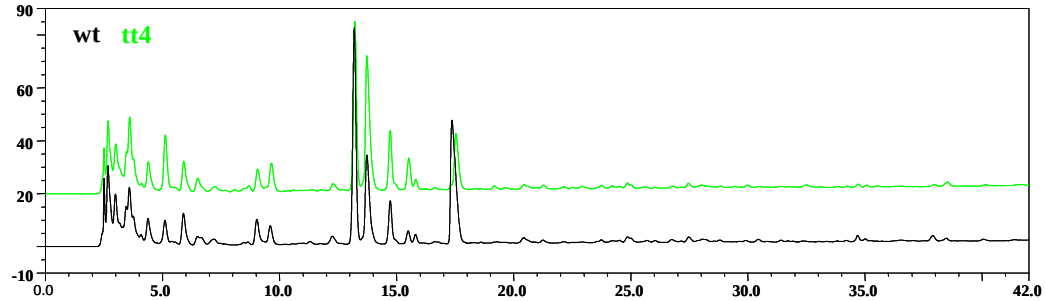
Figure 49 A shows the overlay of the HPLC profiles of the different standards. Most of them have a retention time comprised between 22 and 28 minutes. The seed profiles show strong differences between wild-type and *tt4* mutant (Figure 49 B), as expected from the color of seeds (*tt4* seeds are yellow, while wt seeds are brown). The major compounds lacking in the mutant eluted between 22 and 29 minutes, while some phenolics, with a shorter retention time, were present in higher amounts in the mutants (arrows), indicating that increased amounts of simple phenolics were the result of inhibited flavonoid synthesis.

In leaves, the differences between the two genotypes are less evident (Figure 49 C), but many phenolic compounds are present in lower amounts (arrows) in the leaves of the mutant. Interestingly, except for the two peaks eluting at 24 min, which are also present in seeds, most of the phenolics observed in the seeds are absent in the leaves. In contrast, more hydrophilic compounds (eluting during the first ten minutes) are found in the leaves, compared to the seeds.

The profiles in the roots were quite similar in the mutant and the wild-type, for the internal contents (Figure 49 D) as well as for the secretion (Figure 49 E). This may be due to the absence, or the very low amounts of flavonoids in the roots of *A. thaliana*, reflected by the absence of compounds with a retention time higher than 20 min. However, in the secretion profile (E), some small peaks were detected in the wt, which were even smaller or lacking in the mutant (filled arrows) and in contrast, some peaks were higher in the *tt4* plants (empty arrows).

HPLC profiles of phenolic compounds of *A. thaliana* wild type and *tt4* mutants

D. Roots: internal contents



E. Roots : secretion

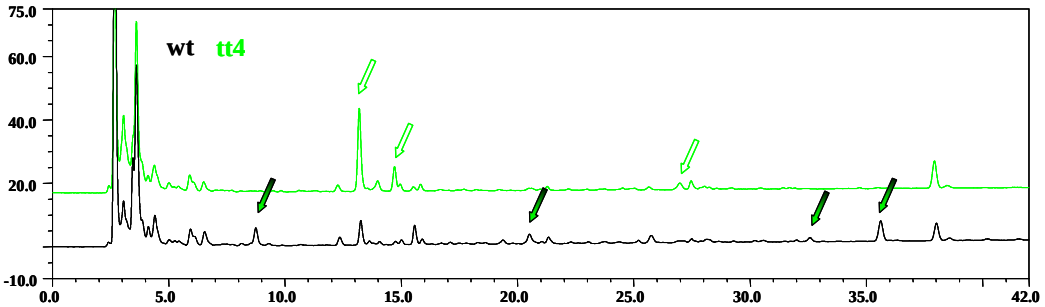


Figure 49 (cont.): HPLC chromatograms of phenolic compounds in different plant parts of* A. thaliana *wild-type (wt) and* transparent tes*ta 4 mutant (*tt4*).

Analysis was performed in 40 min on a C18 reversed-phase column with an increasing acetonitrile gradient. Absorbance was measured at 263 nm. D. Phenolic compounds extracted from root internal contents in wt and tt4 *plants. E. Phenolic compounds secreted in one hour incubation in water by roots of wt and* tt4 *plants.*

Since our extraction and HPLC profiling methods did not allow us to see differences in flavonoids at the root levels, we decided to perform an *in situ* coloration of flavonoids with a DPBA staining. Figure 50 A shows the staining result for whole seedlings. From this picture, it can clearly be seen that wild-type plants produce more flavonoids than *tt4* mutants, which is a logical consequence of the lack of chalcone synthase activity. This was then confirmed by observing root sections under the UV light in the microscope (Figure 50 B). wt roots (upper picture) showed a yellow color after DPBA staining,

which characterizes the presence of flavonoids. Flavonoids were not homogeneously distributed in the root tissues, but had formed vesicle-like structures (middle picture).

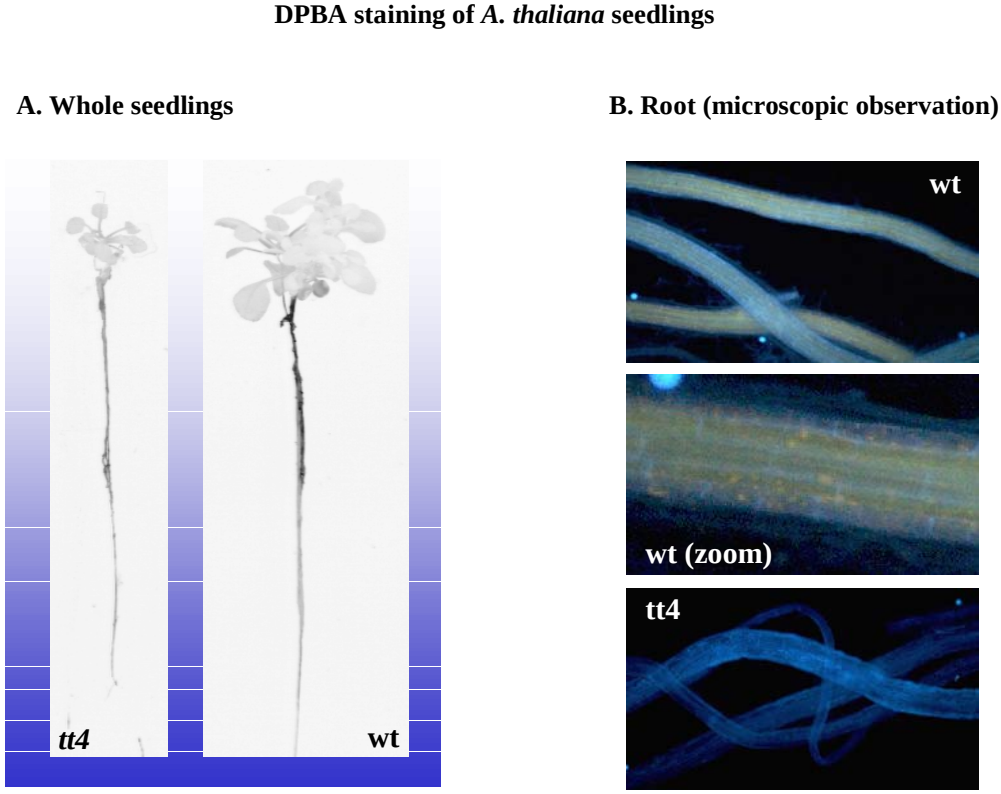


Figure 50: **In situ *flavonoid staining with DPBA (diphenyl-diamino-mehthylesther boric acid).***

A. Staining of whole seedlings of wt and tt4 *plants after 2 weeks growth on plates with ½ MS medium. Staining was performed by placing the roots between two agarose gel layers containing 5 ‰ DPBA. After 30 min incubation, seedlings were observed on an UV plate. B. Microscopic observation under UV light of root sections of wt and* tt4 *seedlings. Staining procedure was the same as for A.*

The roots of the *tt4* mutant (lower picture) were very clearly deprived of flavonoids, as demonstrated by the absence of the yellow color. Therefore, even if the HPLC profiles did not give us a good representation of flavonoid contents in the roots of wild-type and *tt4 A. thaliana*, with the DPBA staining and the following microscopic observations, we were able to clearly differentiate between the two genotypes.

Bacterial communities associated with wild-type and tt4 A. thaliana

Plants were grown in soil in a green house and after 6 weeks, the phenotype of the mutant is even more visible (Figure 51) than in hydroponic cultures: leaves of wt are not only larger, but show the typical violet color indicating anthocyanins, whereas the mutant's leaves are smaller and green, turning to yellow. This enhanced phenotype could come from the higher amounts of UV light contained in the sun light, as compared with the ones used in the growth chambers (hydroponic cultures). *tt4* plants also were a little bit more advanced in their developmental stage (yellow siliques).

Figure 51: wt and* tt4 *in pots

For the microbiological analyses, wt and tt4 A. thaliana *plants were grown in pots filled with soil for six weeks in a green house. Mutants show a typical phenotype after growth with normal sun light: the leaves are smaller and green due to lack of anthocyanin production.*

Form the extracted DNA and RNA, after (RT-) PCR amplification of the v3 fragment of the 16S rRNA gene, separation of the PCR products was achieved by DGGE. Figure 52 shows one example of the four replicates gels which were run and which contained one replicate sample of all the fractions studied: the first four samples come from the *tt4* rhizosphere (D: DNA, R: RNA, RE: root, RS: rhizosphere soil), the following four from the wt rhizosphere. The two bulk soil samples are controls and give an idea of the initial microflora present in the soil used for plant growth.

DGGE profiles based on 16S r RNA gene polymorphism of present and active bacterial communities in the rhizosphere of *A. thaliana* wild-type and *tt4* mutants

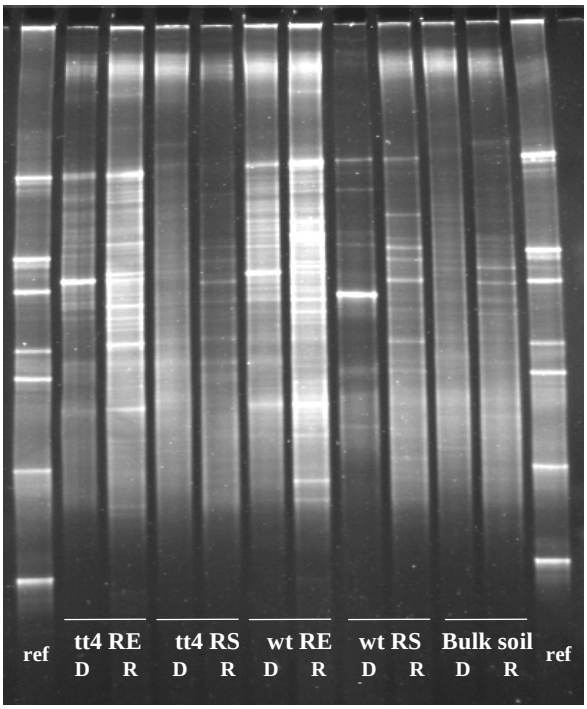


Figure 52 DGGE profiles obtained from amplified v3 segments of the 16S r RNA gene.

Samples from the two different genotypes (wt and tt4*), from rhizosphere soil (RS) and root (RE, rhizoplane, endorhizosphere) and from RNA (R) and DNA (D) are shown. This gel is one representative example of four replicates. The two references lanes (ref) show the v3 amplified segments of seven collection strains differing in their GC content.*

On this DGGE gel, one can already see that, as expected, root samples differ greatly from soil samples, but also within a same fraction (root or soil), differences can be observed between the two genotypes. To better analyze and quantify these differences, we made a cluster analysis, calculated the richness of the different communities and performed a correspondence analysis of the obtained profiles.

Cluster analysis and richness of the bacterial communities

DGGE profiles of present and active bacterial communities in the rhizosphere of *A. thaliana* wild-type and *tt4* mutants

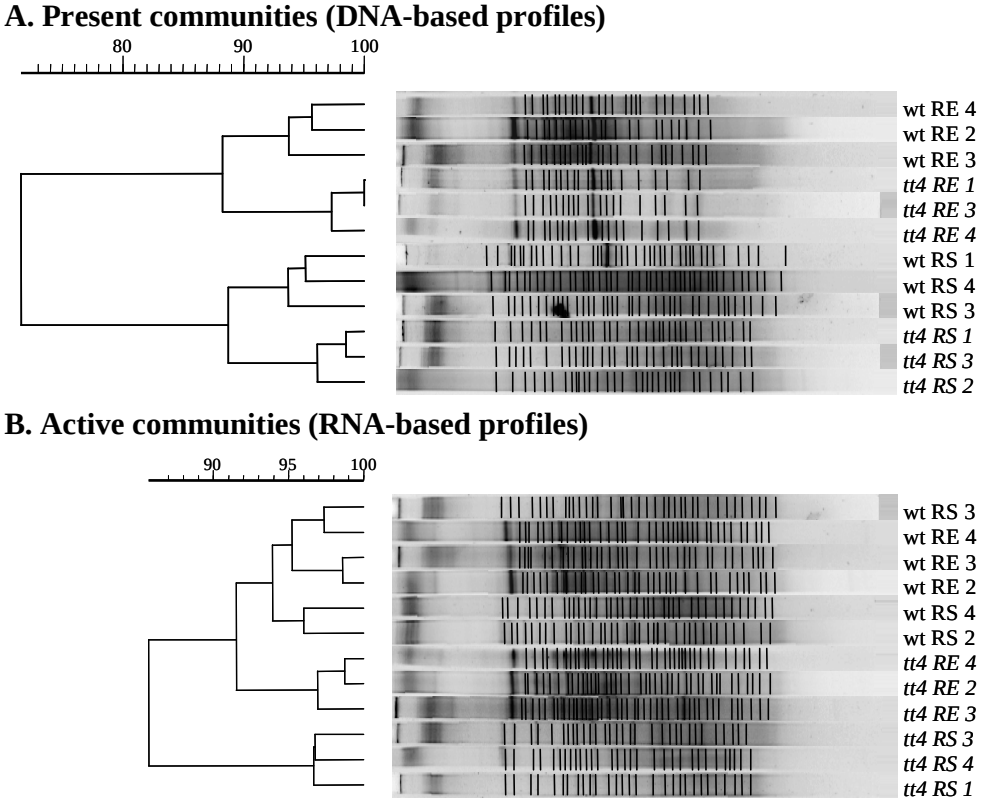


Figure 53: Clustering analyses of the DGGE profiles for the two plants (wt and* tt4*) and for the rhizosphere soil (RS) and the root (RE).

Clustering analysis was performed according to Dice coefficient and the UPGMA clustering method. The scale represent the similarity between samples (in %). A. Present communities (DNA-based profiles). B. Active communities (RNA-based profiles).

Cluster analysis showed a very clear separation (about 70 % similarity) between root and soil samples for DNA-based profiles (Figure 53 A). Interestingly, this was not true for RNA-based profiles (Figure 53 B), where samples were separated according to the genotypes, with the rhizosphere soil of *tt4* plants most distantly related to the other samples. The similarities between rhizosphere soil and root samples within each genotype

were much higher in the RNA-based profiles (about 90 %) than in the DNA-based profiles (about 70 %), while the similarities between genotypes were approximately the same in both types of profiles (85-90 %). This indicates that for active communities, the dominant populations are more similar in the soil and the roots, as for present communities, where differences between soil and root are larger.

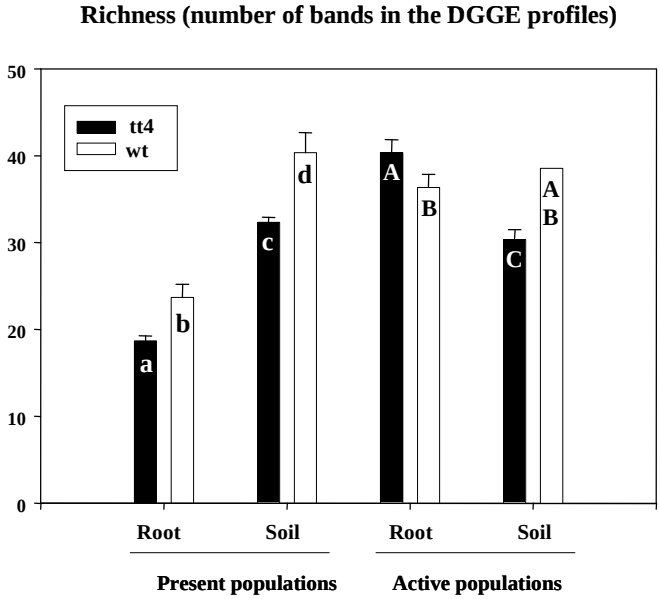


Figure 54: Richness values for present and active communities associated with the rhizosphere soil (RS) and the root (RE) of wild-type (wt) and mutant (tt4) plants.

Bars are averages of three replicates, except for the active soil populations of the wt (2 replicates). Samples are compared within the same type of community (present or active). Letters (a, b, c and d) stand for significantly different values (Student's t test, $P < 0.05$, $n = 2, 3$).

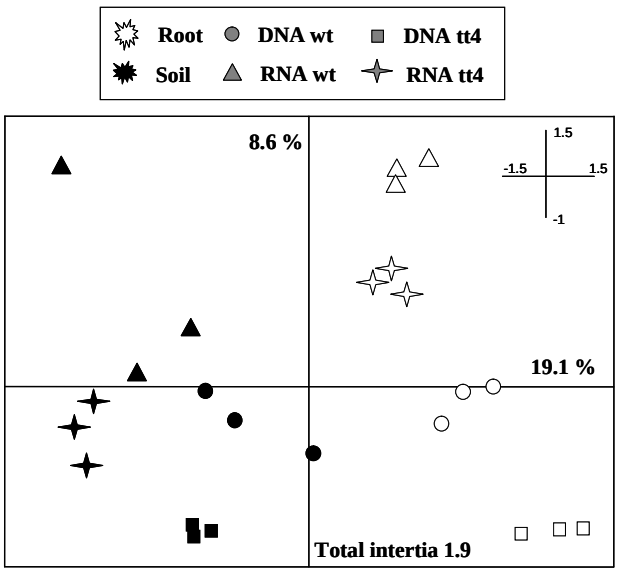
Community richness of the different samples is shown in Figure 54. For present communities (DNA-based profiles), higher richness values were found for soil samples than for root samples, irrespective of plant genotype. This is not surprising and can be related to the selective and elective effect of root secretion on the bacterial microflora of the rhizosphere. The opposite tendency was observed for active communities: as expected, a higher richness characterized the root-associated communities than the soil-associated communities, suggesting that in the root vicinity, bacterial populations are generally more active. In both the root and soil samples, present communities associated with *tt4* plants showed a lower richness than those associated with wild-type plants. For active communities, a higher richness was found in *tt4* root samples than in the wild-type, but the opposite trend was observed for the soil samples.

Correspondence analysis of the DGGE profiles

The global correspondence analysis (CA) on all samples is shown in Figure 55 A: a clear separation between root and soil samples could be observed along the first axis, with all root samples (white symbols) on the right, and all soil samples (black symbols) on the left. Furthermore, the present and active communities were separated along the second axis, more clearly for the root samples, but also for the soil samples. In the root fraction, the communities associated with wt plants (circles and triangles) and with *tt4* plants (squares and stars) were grouped separately. Interestingly, replicates of root samples were much more grouped together than replicates of soil samples, which may be related to the selective effect of roots in the structuring of associated microbial communities.

Figure 55 Correspondence analysis of the DGGE profiles for samples coming from the rhizosphere soil (black symbols) and root (white symbols).

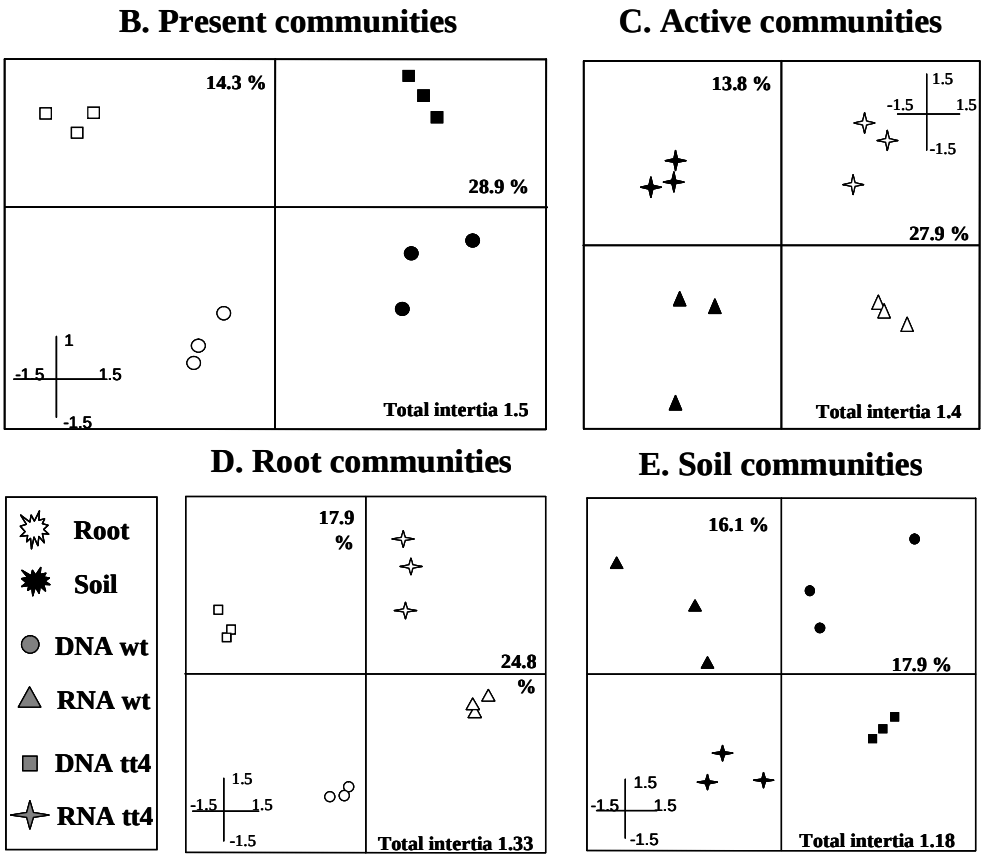
A. Correspondence analysis on total samples



When performing the analyses with only a part of the data set, some interesting additional effects can be shown, when one of the most discriminative factors (root proximity and type of targeted community, active *vs*. present) is eliminated from the analysis. Considering either the present communities only (Figure 55 B) or the active ones (Figure 55 C), the effect of two major factors can be observed: the root proximity on the first axis and the genotype on the second axis.

Figure 55 (cont.): Correspondence analysis of the DGGE profiles for samples coming from the rhizosphere soil (black symbols) and root (white symbols).

*The total inertia is a measure of the total variability between all samples and the percentages of the axes give an idea of the proportion of variability displayed along these axes. DNA samples are represented by squares (*tt4 *rhizosphere) and circles (wt rhizosphere) and RNA samples are represented by stars (*tt4 *rhizosphere) and triangles (wt rhizosphere). A. analysis on total samples; B. analysis on present communities; C. analysis on active communities; D. analysis on root communities; E. analysis on rhizosphere soil communities.*



As for the root communities (Figure 55 D) and the soil communities (Figure 55 E), the type of targeted community – present *vs.* active – is separating the samples along the first axis, while the genotype again is discriminating samples along the second axis.

3.4.5. Discussion

Working with well-characterized mutants to describe rhizosphere processes bears many advantages: it allows to have a proper negative control (wild-type) and to separate the phenomenon studied from all other plant characteristics, even if a single mutation may have many phenotypic consequences and not all them may be known. Moreover, the availability of plant lines containing constructs with reporter genes might be helpful to monitor the changes in gene expression induced by bacterial colonization of the rhizosphere. Although the use of mutants in plant physiology and molecular biology has become a routine technique, ecologists are much less accustomed to use these tools in their studies. This may have several reasons; one of them surely is the mentality gap which often separates physiologists and ecologists. The former are used to apply a scientific approach based on reductionism to very precisely characterize one gene or one function of particular interest, while the latter are more interested in the global processes taking place in an ecosystem as a whole. Fortunately, these gaps tend more and more to be bridged and the use of mutants in ecological studies is likely to become more frequent in the near future. Reports of studies using *A. thaliana* mutants to investigate root-related processes and plant microbe interactions start to appear in the nineties (Fromin et al., 2001; Glazebrook and Ausubel, 1994; Gomèz-Gomèz et al., 1999; O'Gallaghan et al., 2001; Persello-Cartieaux et al., 2001) . The latter was the first to use mutants impaired in flavonoid synthesis, among which also the *transparent testa* mutants, and investigated the *in situ* colonization of the lateral root cracks by rhizobiae in these mutants. They observed that mutants lacking partly or completely flavonoids (*tt3* and *tt4*) were as highly colonized as the wild-type plants and that no differences could be shown between the two genotypes. In the case of Fromin et al. (2001), the *pgm* mutant, impaired in starch metabolism was used to study the effect of a putative alteration of the root exudates composition on the population structure of *P. brassicacearum*. The authors could observe significant changes in the genetic structure of *P. brassicacearum* colonizing either the mutant or the wild-type.

Prior to the present work, we had been studying flavonoid secretion by the roots of white lupin and had investigated possible roles of these secreted phenolics in the control and selection of microbial communities. Since no mutants are available in *Lupinus albus*, we decided to study the impact of flavonoids on the structuring of rhizosphere bacterial communities in *Arabidopsis.* For this, we used the available *tt4* mutant, lacking flavonoids because of a null mutation in the single-copy gene of the chalcone synthase and compared the bacterial communities associated with *tt4* and with the corresponding wild-type plant.

Characterization of the tt4 *mutant*

In order to verify that the *tt4* mutant indeed did not contain any flavonoid, we performed an HPLC analysis of seeds, leaves and roots of plants grown in hydroponic culture. The profiles for the seeds (Figure 49 B) were clearly different for the two genotypes, but differences were less important for leaves, or even for roots (Figure 49 D and E). Since we did not determine the molecular structure of all peaks, the interpretation of these HPLC profiles is delicate, but we still can see that very low amounts of flavonoids, both in the wild-type and in the mutant, are produced and secreted from the roots. This may come from the fact that we probably diluted the signal by analyzing the root as a whole, whereas most of the flavonoids are located in the hypocotyls-root transition and in the root tip (Peer et al., 2001). However, the DPBA staining (Figure 50) showed clearly that there was indeed a difference, which was not seen in the HPLC profile because of technical reasons and dilution effects. Anyway, we should bear in mind that *Arabidopsis* is not secreting large amounts of flavonoids and from this point of view, the relevance of choosing this plant to study the effects of secreted flavonoids in the rhizosphere can be discussed. It would have been much more interesting to have chalcone synthase mutants in *Medicago*, because as a leguminous plant, the wild-type would secrete much more flavonoids and also isoflavonoids. Moreover, the knowledge would be more easily transferable from *Medicago* to *Lupinus* than from a *Brassicaceae* to a *Fabaceae*.

Even if flavonoids are not secreted in high amounts from the roots of *A. thaliana*, there is still a difference, as demonstrated by the DPBA coloration (Figure 50), between the small amounts of the wild-type and the total absence of these compounds in the mutant. Moreover, large quantities are present in the seeds, which may as well play a role in the first steps of root colonization by rhizosphere bacteria.

Bacterial communities are affected by the genotype

The clustering analysis showed that in the present communities (Figure 53 A), large differences between soil and root samples could be observed, whereas the two kinds of samples were more similar in the active communities (Figure 53 B). This was quite surprising, since one usually assumes that the effect of root proximity is to stimulate the activity of the surrounding microflora through rhizodeposition and exudation. According to this, active communities should be more different between the soil and the root than present communities. On the other hand, one could argue that active communities are more influenced by the root exudates and that in the soil and in the root fractions, exudates would stimulate the same populations. In this sense, it is not surprising that root- and soil-associated communities were more similar between each other when considering active communities than when considering present communities. The effect of genotype could be clearly seen in both types of community, with a similarity of 80 to 90 % between the two genotypes, whereas samples from a same genotype (replicates) shared about 95 % similarity.

These differences between the two genotypes could also be observed when considering the richness of the communities (assessed by the number of bands per profile). Irrespective of the samples compared, a significant difference could always be observed between *tt4* and wt (Figure 54). The lower richness of root present communities, as compared to the soil present communities is not surprising and can be viewed as a consequence of the selection by the root of certain populations in its direct vicinity. The opposite situation in active communities, with a higher richness in roots as compared to soil, can also be related to the root impact, this time as a potential stimulator of bacterial

activity: in the root vicinity, more populations become active than in the soil. This result confirms what was already shown in the rhizosphere of lupin (see chapter 3.2.) and indicates that this may be a general effect of any root system rather than a specific effect of white lupin.

The interpretation of the lower richness which characterizes the present communities in both root and soil associated with the *tt4* mutant as compared with the wild-type is not easy. This lower richness would suggest a higher selective effect of the flavonoid-lacking *tt4* plants, which contradicts the hypothesis that flavonoids are involved in the selective and elective structuring of rhizosphere bacterial communities. This effect clearly cannot be explained by the lack of flavonoids and it is probably due to a different – and unfortunately unknown – feature of this mutant.

The correspondence analysis on all samples (Figure 55 A) allowed identifying the major factors accounting for the divergences in community structure between the different samples: mostly influencing the community structure were the root proximity and the type of targeted community, active *vs.* present. In contrast to the situation in the lupin rhizosphere (see chapter 2.2.), the present and active communities were not more similar to each other in the root than in the soil. Overall, the soil communities seemed less well defined than the root communities, where replicates were grouped tightly together. This could again be reflecting the selective effect of roots on the associated microbial communities, which does not allow as much variability as in the less influenced rhizosphere soil or it could be due to the fact that soil samples are more heterogeneous than root samples. The effect of the genotype, which could not be observed on the global correspondence analysis with all samples, because it was hidden by more important factors such as root proximity or type of community, became visible in the analyses performed in sub-groups of the data set. Figure 55 B and C showed that in both the present and the active communities, samples were located according to root proximity (first axis) and to genotype (second axis). When root and soil communities were analyzed separately (Figure 55 D and E), the type of community, active *vs.* present, became the first factor influencing the data dispersion (first axis) and again, the second factor was the

genotype (second axis). As already observed in the global analysis, the root samples grouped more closely together and the effect of genotype was more marked than in the soil, especially for present populations. This indicated that bacterial communities living in the root tissues and in the root's direct vicinity were more affected by a genotypic change in the plant than the communities living in the surrounding soil, which was not surprising.

Overall, this study showed that the bacterial communities were influenced by the plant genotype, when comparing a flavonoid-deficient mutant with the corresponding wild-type. However, our results did not allow us to conclude that the effects observed are due to the lack of flavonoid secreted into the rhizosphere, since the secretion of flavonoid was not clearly demonstrated in the wild-type and since some observations (decreased richness in *tt4*) could not be explained in terms of elective and selective effects of flavonoids. Clearly, there must be some other mechanism involved in the structuring of rhizosphere bacterial communities. This stresses the importance of a good understanding of the plant studied and of the possible pleiotropic effects of a single mutation. In the case of *tt4*, it is known for instance that the plants show an enhanced transport of auxin from shoots to roots and a consequent release of this phytohormone into the rhizosphere (Buer and Muday, 2004). This altered auxin distribution seems also responsible for the particular morphology of *tt4* roots, which are shorter than the wild-type roots. However, it remains unclear to what extend this auxin secretion and root morphology could influence the surrounding microflora. Flavonoids play major roles in plants, ranging from protection against UV-light to the defense against herbivores, from the regulation of auxin transport to plant microbe interactions. Thus, indirect effects of flavonoid-controlled processes may also well contribute to the structuring of the rhizosphere bacterial communities.

3.5. WHITE LUPIN HAS DEVELOPED A COMPLEX STRATEGY TO LIMIT MICROBIAL DEGRADATION OF THE SECRETED CITRATE REQUIRED FOR PHOSPHATE NUTRITION

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3.5.1. Abstract

White lupins respond to phosphate deficiency by producing special root structures called cluster roots. These cluster roots secrete high amounts of organic acids into the rhizosphere, mostly citrate and malate, which act as phosphate solubilizers and enable the plant to grow on soils with sparingly available phosphate. The success and efficiency of such a P acquisition strategy strongly depends on the persistence and stability of the organic acids in the soil, a parameter which is influenced to a large extent by biodegradation through rhizosphere bacteria and fungi. In the present work, we show that white lupin roots use several mechanisms to reduce microbial growth. The abundance of bacteria associated with cluster roots was decreased at the mature state of the cluster roots, where a burst in organic acid secretion and a drastic pH decrease is observed. Secretion of phenolic compounds, mainly isoflavonoids, induced fungal sporulation, indicating that vegetative growth and thus potential citrate consumption, is reduced. In addition, the activity of two antifungal cell wall degrading enzymes, chitinase and glucanase, were highest at the stage preceding the citrate secretion. Therefore, our results suggest that white lupin has developed a complex strategy to reduce microbial degradation of the phosphate chelating agents.

3.5.2. Introduction

In nature, plants often have to cope with nutrient deficiency. To face this problem, they have developed several mechanisms. In the case of phosphate deficiency, plants may establish mycorrhizal associations, express high affinity phosphate transporters or secrete large amounts of organic acids such as citrate or malate. Secretion of organic acids has been reported for many plants in response to phosphate deficiency (Imas et al., 1997; Kirk et al., 1999; Zhang et al., 1997). In addition to this mechanism, some plants like white lupin or many members of the Proteaceae family form special root structures called cluster or proteoid roots (Dinkelaker et al., 1995; Lamont, 2003; Neumann and Martinoia, 2002; Purnell, 1960). These bottle brush-like roots strongly acidify the surrounding rhizosphere and secrete large amounts of organic acids, mainly citrate, during a short time span (three to five days). This strategy enables the plant to efficiently extract Pi from a restricted volume of the soil. Protons solubilize Pi in calcareous soils, whereas di- and tricarboxylates act as exchanger anions for rock phosphate, liberating phosphate from Fe-Al-P complexes. This secretion of protons and organic acids allows plants forming cluster roots to survive on soils with sparingly available phosphate. However, the efficiency of organic acid secretion in phosphate acquisition depends to a large extent on the stability and persistence of these compounds in the soil, a parameter which is mainly influenced by the biodegradation activity of soil microorganisms. Bacteria and fungi are known to readily take up and metabolize organic acids (Jones et al., 1996; Ström et al., 2001; van Hees et al., 2005). Thus, for plants, an efficient strategy in phosphate acquisition would require either an over-secretion of carboxylates to compensate for the loss by microbial degradation and/or a strategy serving to limit microbial degradation of organic acids The second strategy would imply that plants implement mechanisms reducing growth and viability of microorganisms in the vicinity of cluster roots. Among the numerous studies devoted to cluster roots and their efficient phosphate acquisition mechanisms, no report up to now has addressed the question whether or not plants also evolved strategies to protect their secreted phosphate-chelating agents from microbial degradation.

We chose to investigate this question by using white lupin as a model plant. This leguminous annual crop has often been used during the last years to study processes linked to cluster root function (Gardner et al., 1982b, 1982c, 1983; Johnson et al., 1994; 1996a, 1996b; Langlade et al., 2002; Neumann et al., 1999, 2000; Penaloza et al., 2002; Shane et al., 2003b; Shen et al., 2005; Zhang et al., 2004). Formation of cluster roots follows a well-defined developmental pattern (Massonneau et al., 2001). Young, growing cluster roots release mainly malate and only low amounts of citrate while immature cluster roots secrete similar amounts of citrate and malate. In contrast, mature cluster roots secrete far higher amounts of carboxylates, mainly citrate and strongly acidify the rhizosphere. Solubilization, soil extraction and uptake of phosphate into the plant occur mainly at this mature stage of cluster root development. Hence, mechanisms developed for the protection of secreted organic acids against microbial degradation should be observed mainly at this root stage or shortly before.

The aim of this study was to investigate if white lupin has developed strategies to protect secreted organic acids from microbial degradation. We could show i) a decreased abundance of total bacteria around mature cluster roots, ii) an increased isoflavonoid secretion inducing fungal sporulation in the juvenile and immature stage and iii) an increased activity of glucanase and chitinase at the stage preceding the secretion of citrate. Our results suggest that white lupin has developed a complex strategy to protect secreted organic acids against microbial degradation.

3.5.3. Material and methods

Plant material and growth conditions

White lupin plants (*Lupinus albus* L. cv. Amiga, Südwestdeutsche Saatzucht, Rastatt, Germany) were grown either in microcosms (microbiological analyses) or under hydroponic conditions (phenolics, enzymatic analyses). Microcosms were used in three replicates consisting of one plant planted in one microcosm. Plants were grown on inoculated sand as previously described (Weisskopf et al., 2005), except that the

inoculation solution was prepared from a lupin field soil collected in Gargano (Italy). After 6 weeks growth, plants were harvested and the different root stages identified with a pH indicator – agar gel method (Weisskopf et al., 2005). Juvenile, mature and senescent stages were separated (in soil-grown plants, the distinction between immature and mature cluster roots is not possible). Within a plant (replicate), cluster roots belonging to the same stage were pooled. Roots coming from the different root stages were then washed in a sodium-phosphate buffer 0.1 M pH 7 (SPB) and ground with mortar and pestles. From the ground roots, one part was used for isolation and low pH tolerance assay of bacterial strains and the other was prepared for the microscopic count analysis. For phenolics and enzymatic analyses, growth in hydroponic cultures without phosphate supply was carried out as described by Massonneau et al. (2001). Three replicates were used and one replicate consisted of about five boxes containing each 12 plants. The different cluster root growth stages were identified by immersing the root system into a pH-indicator solution according to Neumann et al. (1999). This technique allows the separation between immature and mature roots; therefore we were able here to collect separately juvenile, immature, mature and senescent cluster roots.

Isolation of strains and growth in low pH media

Ground roots coming from the three different stages of cluster roots were tenfold-serially diluted and spread on Angle medium (Angle et al., 1991) for plate counts and isolation of strains. Approximately 30 strains isolated from each cluster root stage were randomly selected and tested for their ability to grow in low-pH media. We prepared LB medium (10 $g.l^{-1}$ peptone, 5 $g.l^{-1}$ yeast extract, 10 $g.l^{-1}$ NaCl) and modified the pH to obtain five different pH values, from 7 to 3. For each pH value, a specific buffer was used. NaOH 0.1 M (A), $C_8H_5KO_4$ 0.1 M (B), HCl 0.1 M (C) and water (D) were used for the buffers in the following proportions (A:B:C:D): 45:50:0:5 for pH 6, 22.6:50:27.4:0 for pH 5, 0:50:0:50 for pH 4 and 0:50:21.6:28.4. 10 ml of the corresponding buffer was added to 250 ml of medium prior to autoclaving. The low pH assay was performed in liquid cultures, using micro-plates (NUNC^{TM}). Results (growth or no growth) were recorded after seven days incubation at room temperature.

Total cell counts after DAPI staining

After washing off the rhizosphere soil by gentle shaking in SPB, roots coming from the three different stages of cluster roots were ground. One ml of the ground roots were added to nine ml of ethanol 50 % for fixation. Sampled were incubated for four minutes in an ultrasonic bath for sample homogenization, final separation of roots from remaining soil and sand particles and partial dissociation of bacteria from roots. Several incubation times were tested and the highest recovery of bacteria was obtained after 4 min of sonication. Samples were then centrifuged for two min at 2000 rpm to allow precipitation of remaining soil and sand particles. After diluting 1:1 with ethanol 50 %, one ml of the supernatant was filtrated on polycarbonate filters (diameter 13 mm, pore size 0.2 µm) placed on nitrocellulose filter supports (diameter 13 mm, pore size 0.2 µm). Samples were then stained with 70 µl DAPI (4'-6-Diamidino-2-phenylindole, SIGMA, 5 $\mu g.ml^{-1}$ solution) for 5 minutes in the dark. Excess DAPI was removed and filters were observed with a Leica Dialux microscope at a 1000x magnification. Cell numbers were determined by counting 15 fields per filter with a grid ocular. The average value of the 15 fields was used for calculation of the total cell counts g root fresh $weight^{-1}$.

Extraction of phenolic compounds and HPLC analysis

Excised root parts were washed in distilled water to eliminate the compounds liberated from cut cells and subsequently incubated in four ml water for one hour at room temperature under gentle shaking to allow the collection of root exudates. We have previously shown that there was no significant difference in quality or quantity of exudates released into distilled water, as compared with $CaSO_4$ 0.5 mM, which is often used for membrane stabilization (Neumann et al., 1999). Even if the secreted compounds recovered during this collection procedure might be slightly different from those obtained in the field conditions, this experimental protocol is reproducible and allows the comparison of well-defined cluster root stages. The root exudates were collected and frozen at -80 °C. The remaining roots were then incubated in four ml methanol 80 % for one hour at room temperature under gentle shaking to recover the internal cell contents.

Internal content extracts were filtrated at 0.45 µm (Schleicher & Schuell) and resuspended in the first HPLC solvent according to the root fresh weight (1.5 µl mg root fresh weight $^{-1}$). The frozen exudates were freeze-dried and subsequently extracted with 2.5 ml methanol 80 % in sequential steps (1 ml and subsequently three times 0.5 ml). Each step was followed by vigorous shaking and filtration at 0.45 µm. After solvent evaporation, exudate extracts were resuspended in the first HPLC solvent in proportion to the root fresh weight (0.75 µl mg root fresh weight $^{-1}$). 50 µl of the samples (internal contents and exudates) were then injected into a reversed phase C 18 column (NUCLEOSIL 250 x 4.6 mm, 7.0 µm) for analysis. To separate the isoflavonoids, we used two solvents, consisting of water, acetonitrile and acetic acid in the following proportions: 93:5:2 (A) and 23:75:2 (B). Solvent gradient started with 10 % solvent B and reached 100 % B in 25 minutes, with a flow rate of 0.4 ml.min^{-1}. Absorbance was monitored at 263 nm. All analyses were performed with 3-4 replicates, each replicate representing the harvest of about five boxes containing each 12 plants.

Flavonoid staining with DPBA

The entire root system of white lupin plants grown in hydroponic culture in P deficient conditions was placed between two agar gel layers containing 5 ‰ of diphenyl-boric acid dimethyl amino-esther (DPBA, SIGMA). After 20 minutes incubation in the dark, the stained root parts were revealed under UV light (360 nm) exposure. The same staining procedure was applied for single roots.

Effect of isoflavonoids on the sporulation of Fusarium

Phenolic compounds (100 µg in ethanol in 20 µl ethanol 50 %) were separated on TLC plates (Silica gel 60 F254, Merck, Darmstadt, Germany) in a dichloromethane/methanol mixture (95:5) and one half of the plate was used for isoflavonoids staining with 1 % Gibbs reagent in ethanol. The solvent (ethanol 50 %) was used as a control. A spore suspension of the lupin pathogen *Fusarium oxysporum* was spread on the other half of the plate. The spore suspension was prepared by mixing 1 ml of sterile water containing

spores collected on a Petri dish and 10 ml of melted PDA (potato-dextrose-agar) tenfold diluted and containing half the agar concentration to allow a thin and easy spreading on the plate.

Enzymatic assays of chitinase and glucanase

For the analysis of enzymatic activities, plants were grown in hydroponic culture in P deficient conditions. Four to seven replicates were used, consisting of one box containing 12 plants. After revelation of the different cluster root stages, excised root samples were incubated for 30 min at room temperature in $CaCl_2$ 5 mM (pH 6.4) under gentle shaking. The extracellular fraction was collected, 1 mg ml^{-1} BSA was added and samples were frozen at -80°C prior to lyophilization. Lyophilized samples were resuspended in a 0.1 M sodium acetate buffer pH 5.

Chitinase activity

Chitinase activity was determined with dye-labelled CM-chitin (chitin azure, SIGMA) according to Wirth and Wolf (1990) after optimization of incubation time and enzymatic extract concentration. Each reaction mix contained 0.2 mg chitin azure, 500 µl enzymatic extract and 200 µl 0.1 M sodium acetate buffer pH 5. For each sample, a blank sample containing no chitin azure was also prepared to take into account the colour differences of the different root stages. After one hour incubation at 30°C and shaking at 150 rpm, 200 µl HCl 0.1M were added to stop the reaction and samples were centrifuged for 5 min at 14000 rpm. Absorbance was read at 560 nm. For each sample, the absorbance value of the negative control (lacking chitin-azure) was deduced from the final absorbance. Chitinase activity was expressed as the absorbance at 560 nm root fresh $weight^{-1}$ h^{-1}.

Glucanase activity

Glucanase activity was determined by measuring the release of glucose from laminarin. Each reaction mix contained 100 µl of laminarin solution (18 mg ml^{-1} in sodium acetate

buffer pH 5 0.1 M) and 200 µl enzymatic extract. For each sample, a blank sample containing no laminarin was used to account for the colour changes between different root ages. After one hour incubation at 30 °C and shaking at 150 rpm, 150 µl were collected and released glucose was determined with the Boehringer Mannheim D-Glucose determination kit. Glucanase activity was expressed as mg glucose root fresh weight^{-1} h^{-1}.

Statistical analyses

Microscopic counts were compared statistically using the Student's *t*-test ($P < 0.01$). Results of bacterial growth in low pH media were validated using a χ^2-test ($P < 0.05$). For phenolic contents and enzyme activities, the Student's *t*-test was used to validate differences ($P < 0.05$). These analyses were performed using S-Plus 6 Statistical Software (Insightful Corporation, Seattle, WA, USA).

3.5.4. Results

Acidification at the mature cluster root stage decreases bacterial abundance

In a recent paper (Weisskopf et al., 2005), we showed that the number of cultivable bacteria was significantly reduced at the mature state of cluster roots. In order to verify if this reduced abundance deduced from plate counts was not due to a decrease in cultivability, we performed microscopic counts after DAPI staining of bacteria coming from different root stages. As already observed for cultivable bacteria, total bacterial abundance decreased significantly at the mature stage of cluster roots, which corresponds to the stage of highest citrate secretion (Figure 56).

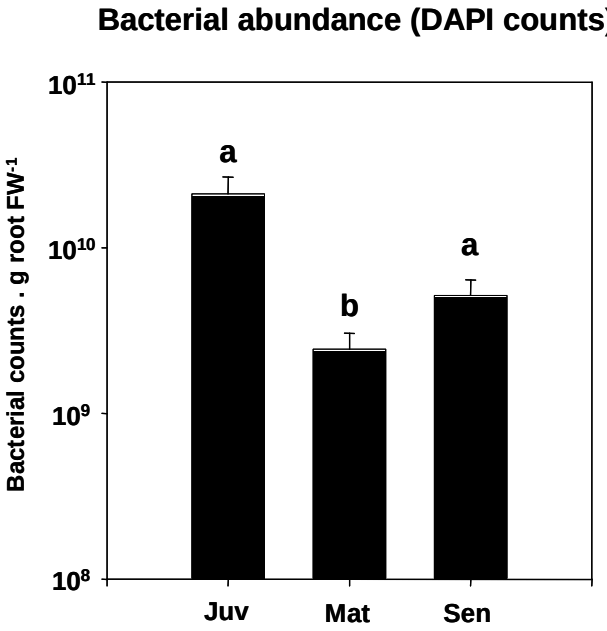


Figure 56: Bacterial abundance

Numbers of bacteria g^{-1} dry weight in function of root stage. Bacterial abundance was determined by microscopic counts after DAPI staining. Juv: juvenile, Mat: mature, Sen: senescent. Values with different letters (a, b) are significantly different (Student's t-test, $P < 0.01$, $n = 3$).

A possible explanation for this decrease could be the low pH (4 or below) associated with the mature stage of cluster roots, due to a concomitant release of protons and citrate secretion. To test this hypothesis, we assessed the ability of isolated strains to grow at pH conditions ranging from pH 3 to pH 7. The ability of about 100 strains isolated from three stages of white lupin's cluster roots to grow on an acidic medium (pH 4) is shown in Figure 57. The proportion of strains able to grow at pH 4 was significantly higher for mature cluster roots than for the juvenile stage, suggesting that due to the transient

acidification of the rhizosphere, a selection of acid-tolerant populations had taken place at the mature stage of cluster roots, whereas the more sensitive populations had been inhibited. At pH 3, no bacterial growth was observed and no significant differences between the different stages were observed at the other pH values (data not shown).

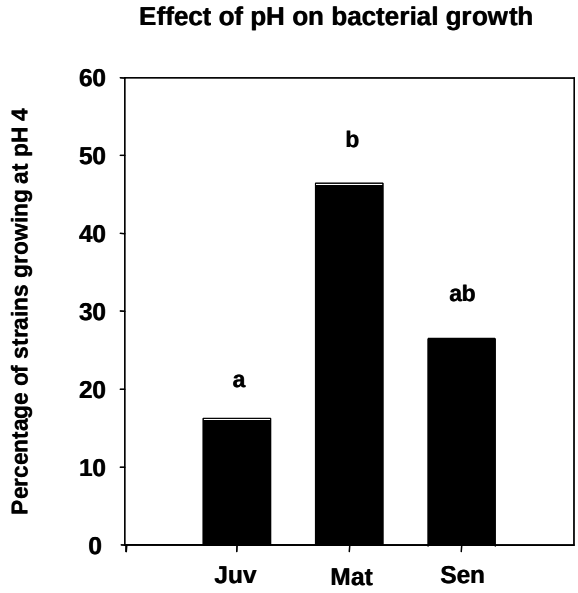


Figure 57: Effect of pH on bacterial growth

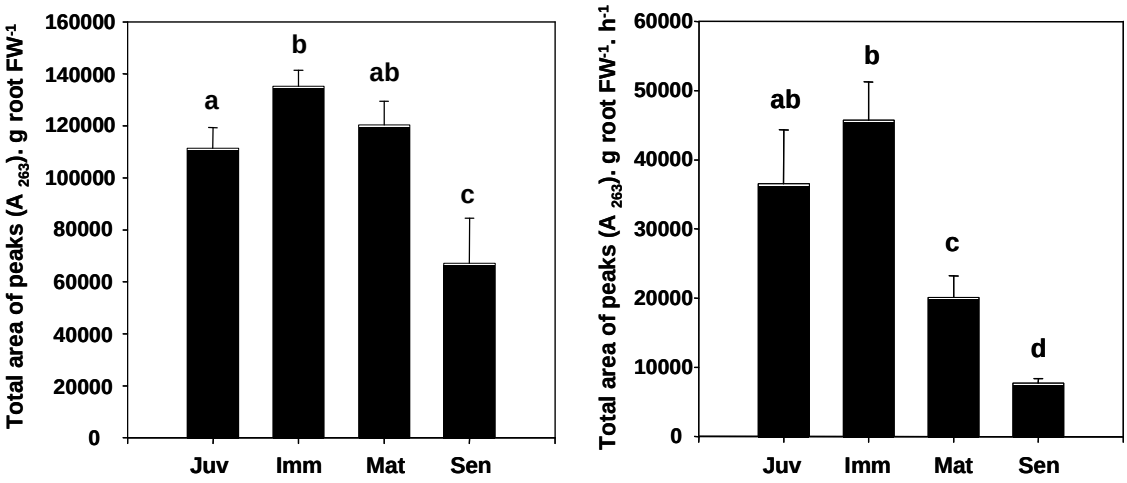
Frequencies (in % of total isolated strains) of strains growing in a low pH (4) medium. About 100 strains were randomly picked, at least 30 per root stage. Values with different letter (a, b) are significantly different (χ^2-test, $P < 0.05$, $n \geq 30$).

Prior to citrate secretion, isoflavonoids are secreted in high amounts into the rhizosphere

Since fungi are known to be more tolerant to acidic pH than bacteria, we investigated other possible defense mechanisms involved in fungal growth inhibition. It has been previously reported that cluster roots secrete phenolics (Neumann, 1999), which might act as antifungal compounds, and this is the reason why we investigated this class of compounds in more detail. White lupin cluster roots secreted large amounts of phenolic compounds and most of these phenolics were isoflavonoids. The internal content of isoflavonoids significantly increased from the juvenile to the immature stage (Figure 58 A). The secretion pattern followed the same tendency, with the highest amounts secreted at the juvenile and immature stages, the root stages preceding the citrate burst (Figure 58 B). Genistein was the isoflavonoid secreted in highest amounts (up to 2 mg g^{-1} root fresh weight in immature cluster roots, data not shown), and most other compounds were conjugates of genistein (Weisskopf et al., unpublished).

Figure 58: Flavonoids in white lupin's cluster roots.

A. Flavonoid contents in cluster roots **B. Flavonoid secretion in cluster roots**



Flavonoids produced in (A) and secreted from (B) different stages of cluster roots. Juv: juvenile; imm: immature; mat: mature; sen: senescent. Total amounts were calculated as the total area of all peaks measured by UV-detection (263 nm) after HPLC analysis. Values are means of three replicates, one replicate consisting of five boxes containing 12 plants each. Different letters (a, b, c, d) represent significantly different values (Student's t-test, $P < 0.05$, $n = 3$).

This pattern of isoflavonoids in the different stages of white lupin's cluster roots was confirmed by an *in situ* staining of the entire root system with diphenyl-boric acid (DPBA), a flavonoid specific dye (Figure 58 C). Young and immature cluster roots exhibited a brighter fluorescence than mature or senescent cluster roots. This was also true for non cluster roots, where mostly the apex and growing parts were stained.

Figure 58 (cont.): Flavonoids in white lupin's cluster roots.

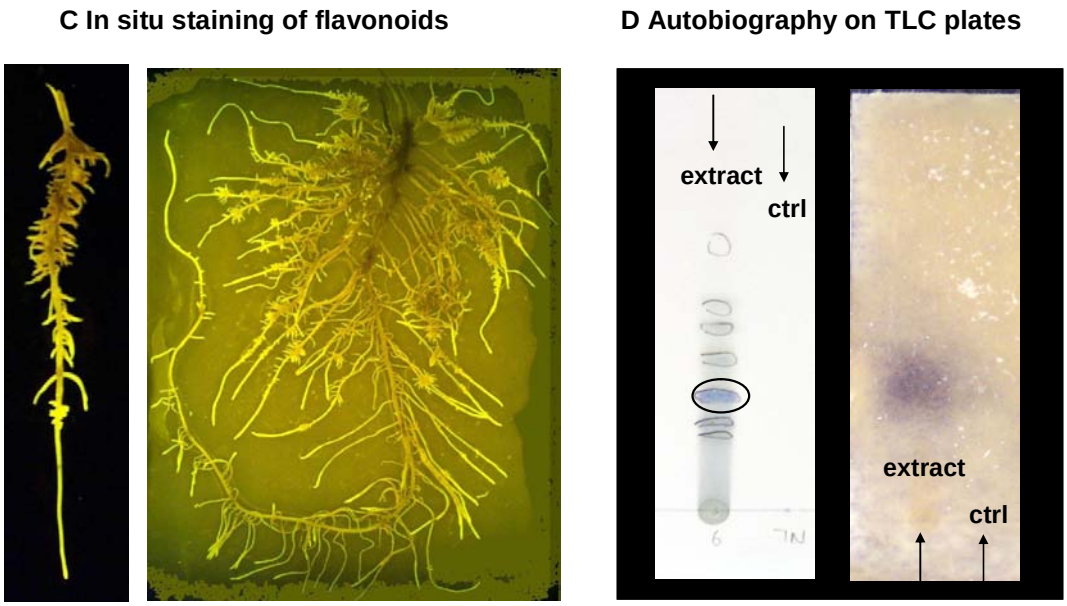


Figure 58(cont.) C In situ staining of flavonoids with DPBA (diphenyl-boric acid dimethyl amino-esther). White lupin's roots (single cluster roots: left picture or whole root system: right picture) were placed between two agar gel layers containing 5 ‰ DPBA. The brightness reflects the amounts of flavonoids present in the root tissues and secreted at the surface. After 20 minutes incubation, roots were exposed to UV light (360 nm). D Effect of flavonoids secreted by white lupin's cluster roots on the sporulation of Fusarium oxysporum. *Left panel: 50 µg of phenolic extracts were separated on a TLC plate and stained with the flavonoid-specific Gibbs reagent. One compound (blue) was highly absorbing UV light (circle). Right panel: Inoculation of the TLC plate with a spore suspension of F. oxysporum showed an enhanced sporulation at the same location as the highly absorbing compound, identified as malonyl-genistin. 50 µg of phenolic extracts were used for this assay. Ctrl: negative control (only solvent). Picture was taken five days after inoculation.*

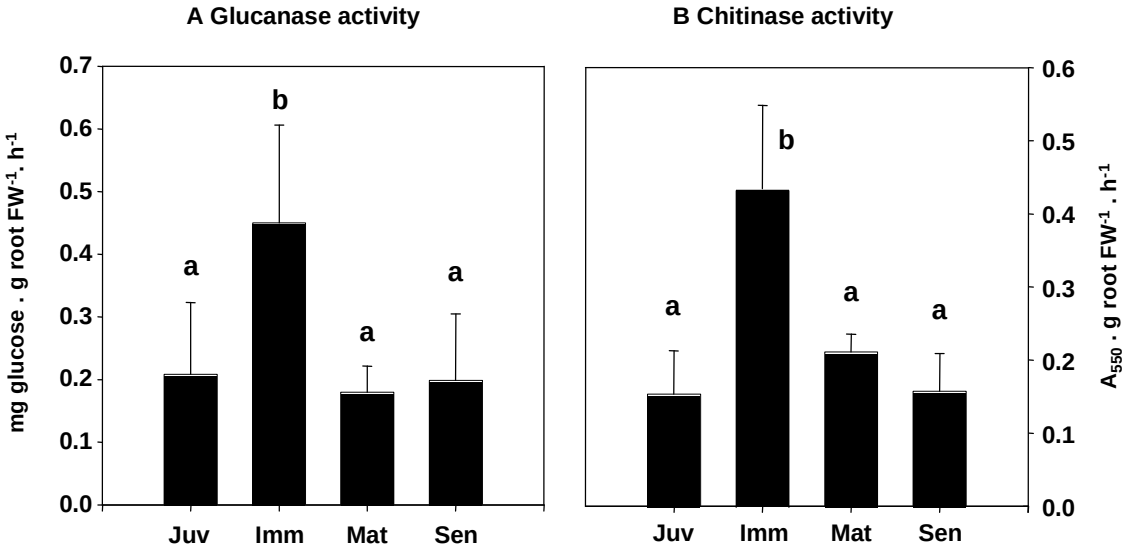
Since isoflavonoids are well-known antifungal compounds, one could imagine that this observed "phenolic burst" prior to citrate exudation would exhibit an inhibitory action on fungal growth and hence also contribute to the protection of citrate from microbial degradation. To test this hypothesis, we performed *in vitro* assays with the bulk of secreted phenolics. We tested about 20 morphotypes of fungi isolated from white lupin's rhizosphere, as well as some collection strains, which represented potentially pathogens

of white lupin. Among the tested fungi, some showed an increased sporulation when exposed to the phenolics secreted by white lupin. Since in fungi, active growth and nutrition occur only during the vegetative state (mycelium) of the life cycle, we can assume that at the sporulated stage, fungi will not consume any citrate. One collection strain, belonging to the species *Fusarium oxysporum*, showed the strongest reaction and was chosen as a bio-test strain for further investigations. By testing purified fractions in a bioassay based on TLC plates bioautography (Figure 58 D), we observed that stimulation of sporulation was localized at the same height as the isoflavonoid with the highest UV absorption, identified as malonyl-genistin. Unfortunately, we were not able to observe this stimulation of the sporulation when fractions were further purified, suggesting that more than one compound could be involved in this phenomenon and that they might act in a synergistic way. No effect of these isoflavonoids on bacterial growth inhibition could be observed.

Chitinase and glucanase show higher activities in the stage immediately preceding high citrate secretion

A wide-spread defense mechanism of plants against a broad spectrum of fungi is the secretion of cell-wall degrading enzymes into the rhizosphere. In a former work we showed that with a cDNA AFLP assay, several genes in carbohydrate metabolism were differentially expressed during cluster root formation in P deficient conditions (Massonneau et al. 2001). Later analysis of the cDNA AFLP data showed that among the genes differentially expressed, there were two antifungal enzymes, a glucanase and a chitinase (not shown), as potential candidates to be highly induced during cluster root formation. To verify that the higher level of gene induction corresponded to an enhanced activity, we measured extracellular glucanase and chitinase activities in the different cluster root stages. Chitinase and glucanase activities were both significantly ($P < 0.05$) higher in the immature stage of cluster roots (Figure 59) compared to all other stages.

Figure 59: Activities of cell-wall degrading enzymes in the different cluster root



Juv: juvenile; imm: immature; mat: mature; sen: senescent. A. Glucanase activity was measured spectrophotometrically by determining the amount of glucose liberated from laminarin during a one hour incubation period. Values are averages of four to seven replicates. B. Chitinase activity was measured spectrophotometrically using the dye-labelled chitin-azure over a one hour incubation period. Values are averages of four to seven replicates. Values with different letters (a, b, c, d) are significantly different (Student's t-test, $P < 0.05$, $n = 4$-7).

3.5.5. Discussion

In natural environments, phosphate is often a limiting growth factor. To deal with this situation, most plants form mycorrhizal associations, which allow the plant to survive on soils with sparingly available phosphate or on soils where phosphate is strongly bound to the soil, mainly as rock phosphate (Fe/Al-P). Still, about 20 % of plant species do not interact with mycorrhizae and among them, some have developed a strategy which is at least as efficient as the mycorrhizal symbiosis: the formation of cluster roots. In contrast to the phosphate acquisition occurring in a broad scale with mycorrhizae, these plants secrete large amounts of organic acids in a restricted volume of soil and a short time span. The organic acids serve as exchange anions to solubilize rock bound phosphate in proximity of the rootlets. The problem of this strategy is that many microorganisms efficiently take up and metabolize organic acids. There was no significant difference between juvenile, mature and senescent cluster root stages when comparing the percentage of isolated strains able to use citrate as a carbon source, but overall, these percentages were very high (85 % on average, data not shown). For plants forming cluster roots (secreting organic acids), it is therefore crucial to limit the breakdown of organic acids by microorganisms. Several mechanisms can lead to an inhibition of microbial growth; however, different strategies have to be used for bacteria and fungi. Bacteria are in general more sensitive to acidic environments than fungi and a transient decrease of pH should limit their density. This transient pH decrease is precisely what happens at the mature stage of cluster roots, which can acidify the proximal environment to a pH of four and below. We could show that the bacterial population levels transiently decrease at the mature stage of cluster roots. Furthermore, the mature stage of cluster roots harbored a significantly higher proportion of strains able to grow in acidic conditions (pH 4) than the other cluster root stages. This indicates that the strong acidification occurring at the mature stage of cluster roots could be responsible for the decrease in bacterial abundance associated with this stage and that only acid-tolerant populations are able to grow in the vicinity of mature cluster roots, whereas the more sensitive populations are transiently inhibited. Thus, the proton extrusion occurring concomitantly to the secretion of organic anions may have several roles: i) maintaining a

charge balance through compensation of the secreted negatively charged citrate, ii) solubilizing P in calcareous soils, iii) providing an optimal pH for the secreted hydrolases, such as chitinase, glucanase or acid phosphatase and, as suggested by our study iv) transiently decreasing the bacterial density in the rhizosphere and reducing their degradation of the phosphate-chelating agents.

Heterotrophic soil fungi also can use organic acids as carbon source and could thus also contribute to a decrease in the P acquisition system efficiency of white lupin's cluster roots. Since fungi usually are better able to cope with acidic conditions, other mechanisms than rhizosphere acidification are required to inhibit fungal growth. It is known that some phenolics, like flavonoids or isoflavonoids, display antifungal activities (Dakora and Phillips, 1996; Tahara et al., 1994; Weidenborner and Jha, 1997). White lupin is known to produce a large amount of different isoflavonoids (Katagiri et al., 2000; Stobiecki et al., 1999) and some of them have been reported to act as antifungal compounds (Bednarek et al., 2003; Wojtaszek et al., 1997). A consistent part of isoflavonoids is secreted from the root. We could show that white lupin isoflavonoids induced sporulation in several fungal strains. Sporulation is often a stress response in fungi and since spores, in contrast to mycelium, do not cause degradation of citrate and malate, this stimulation of sporulation can be viewed as a protection mechanism of the organic acids involved in phosphate acquisition. One could argue that fungi may in turn have a higher nutritional demand for the spore formation and thus may have consumed a lot of organic acids in order to sporulate; however, the fact that the isoflavonoid burst occurs prior to the citrate secretion suggests that sporulation takes place before organic acids are secreted and could be consumed. Interestingly, among the fungi tested, *Fusarium* was the most susceptible to white lupin isoflavonoids. *Fusarium* species have been previously reported to be inhibited by flavonoids (Silva et al., 1998) and to elicit isoflavonoid accumulation in soybean roots (Lozovaya et al., 1994). In addition, *Fusarium* species also are well-known pathogens of lupins (Bateman et al., 1997; Shield et al., 2000; Satyaprasad et al., 2000).

In addition to this increased isoflavonoid secretion, white lupin apparently also uses a second and surely more generally efficient way to inhibit fungal growth: extracellular enzymes like chitinase and glucanase. Both chitinase and glucanase have been shown to be involved in plant defense against pathogenic fungi, in white lupin as well as in other plant species (Burzynski et al., 2000; Tonon et al., 2002; Vierheilig et al., 1994). However, since these enzymes are involved in the fungal cell wall degradation, their inhibitory effect will not be limited specifically to pathogenic fungi, but will also affect other rhizosphere fungi, which may use citrate and malate as a carbon source. In white lupin's cluster roots, we could show that both chitinase and glucanase had a higher activity at the immature stage, the stage preceding the high citrate secretion.

In conclusion, our results suggest that white lupin has developed a complex strategy to protect secreted organic anions from microbial degradation: acidification against bacteria and secretion of isoflavonoids, as well as cell wall degrading enzymes against fungi.

3.6. SYNTHESIS, CONCLUSIONS AND OUTLOOK

3.6.1. Synthesis

Plant microbe interactions play a major role in plant nutrition, growth and health. In order to better understand these interactions in the case of *Lupinus albus* and *Arabidopsis thaliana*, we performed several experiments (chapters 3.2. to 3.5.).

First, we analyzed the bacterial communities associated with different cluster root stages and root proximity and characterized them with respect to their abundance, PGPR functions, diversity and community structure (chapter 3.2.). In this study, we observed important changes in abundance, richness, diversity and functions of the bacterial communities associated with the mature stage of cluster roots. It appeared that cluster roots (considered as a whole, as a "root type") and non cluster roots were not so different from each other, with respect to the associated microflora. In contrast, the bacterial communities living in the rhizosphere of mature cluster roots were reduced in abundance, richness and diversity. This effect of mature cluster roots was not restricted to the immediate vicinity of the roots, referred to as "RE", rhizoplane-endorhizosphere, but extended to the rhizosphere soil ("RS") as well, when considering the decrease in abundance, richness and diversity. The correspondence analysis of the DGGE profiles for both DNA (present communities) and RNA (active communities), based on polymorphism of the 16S rRNA gene, revealed that the most important factors accounting for differences between the communities were i) the root proximity, ii) the type of targeted community (present *vs.* active) and iii) the cluster root stage. This correspondence analysis also showed that larger differences were observed between rhizosphere soil and root fraction in active communities than in present communities. This suggests that bacterial communities living in the root and at its surface were generally more active than bacterial communities living in the rhizosphere soil. The assessment of bacterial properties linked to promotion of plant growth and rhizosphere competence gave the following results: The percentage of siderophore producers was lower in mature cluster roots than in other root stages, suggesting that iron might not be a

limiting factor for bacteria in the vicinity of mature cluster roots. Since phosphate solubilization by the plant occurs to a great extent at this stage and since most of the phosphate is present in a iron-bound form, one can postulate that solubilization of phosphate from iron complexes will also increase the availability of iron, which could explain the lower percentage of siderophore producing bacteria in the rhizosphere of mature cluster roots. Finally, the proportion of auxin producing bacteria was highest for juvenile cluster roots; an interesting feature when considering that auxin is involved in cluster root formation. Overall, our results indicated a strong selective effect of roots, and especially of mature cluster roots, on the surrounding microflora, which was expressed by the decrease in abundance, richness and diversity.

The observed decrease in abundance of cultivable bacteria associated with the mature stage of cluster roots, where the secretion of the phosphate-solubilizing citrate occurs, led to the idea that white lupin might have evolved strategies to protect the organic acids secreted for phosphate acquisition from microbial degradation. We investigated the presence of such protection mechanisms and came to the conclusion that white lupin indeed evolved strategies to transiently lower the abundance of bacteria and decrease the potential degradation of citrate by both bacteria and fungi. We observed that among the strains isolated from mature cluster roots, a significantly higher percentage was able to grow at low pH (4) than among the strains isolated from juvenile and senescent cluster roots. This was an indication that acidification, which is due to the proton release occurring concomitantly to the secretion of citrate in mature cluster roots, could be a way to transiently lower the abundance of the bacterial populations living in the rhizosphere of mature cluster roots and thus to decrease the bacterial citrate degradation rates. Since this decrease in bacterial abundance was also observed in total bacterial counts (DAPI staining), where dead bacterial are also stained, one can assume that the effect of pH is a repulsion (negative chemotaxism causing mobile rhizobacteria to move away from mature cluster roots) rather than an inhibition or killing effect. Since fungi are usually not as sensitive to low pH as bacteria, we investigated other plant mechanisms which could lead to the inhibition of fungal growth. Phenolic compounds, and isoflavonoids in particular, are known to display antifungal activities. As already described in chapter 2,

isoflavonoid secretion occurred in highest levels in immature cluster roots, just before the secretion of citrate at the mature stage. This phenolic burst at the stage immediately preceding the stage of organic acid exudation was an indication of a potential protection mechanism against the microbial degradation of citrate. We performed *in vitro* tests to assess the antimicrobial activity of the isolated isoflavonoids and surprisingly, no effect of isoflavonoids on growth inhibition of bacteria and fungi could be observed. This lack of antimicrobial activity of white lupin isoflavonoids can be related to the absence of prenylated isoflavonoids, which are known to be the most active antimicrobial compounds, especially against fungi (Ingham et al., 1983; Tahara et al., 1984). In some fungal strains, a stimulation of the sporulation could be observed. *Fusarium* species seemed to be the most susceptible to white lupin's isoflavonoids. Fungal sporulation can be viewed as a stress response and with respect to the citrate degradation, sporulation certainly is positive from the plants' point of view, since only the vegetative state of fungi is susceptible to feed and thus to use citrate as a carbon source, while spores merely represent a dormancy status, where no metabolic activity is present. In addition to isoflavonoid secretion, the immature stage was also characterized by a high activity of two antifungal enzymes: a chitinase and a glucanase, which had been previously identified in a cDNA-AFLP analysis as genes differentially expressed during cluster root development. These two cell wall degrading enzymes are likely to inhibit fungal growth in a very broad way, since all fungal species, with the exception of Oomycetes, have a cell wall built of chitin. Altogether, these results indicate that white lupin does not only overproduce and over secrete citrate to compensate for the losses due to microbial degradation, but that it has evolved a complex strategy to protect the secreted citrate from bacterial and fungal consumption: acidification against bacteria and isoflavonoids as well as cell-wall degrading enzymes against fungi. Interestingly, all these mechanisms are transient and occur simultaneously to (acidification) or shortly before (phenolics and antifungal enzymes) the citrate secretion at the mature stage of cluster roots. This indicates that white lupin only inhibits the microbial rhizosphere populations when it is needed and not in a lasting manner, which would represent a waste of energy and the risk of inhibiting potentially beneficial rhizosphere microorganisms.

In addition to the high availability of organic compounds and especially citrate, which is a very good source of carbon for heterotrophic bacteria and requires thus the protection mechanisms described above, another feature of the cluster root environment is that oxygen levels are very low due to a high respiratory activity of the root. Such conditions, high availability of organic carbon and low oxygen pressure, make cluster roots a potentially favorable environment for associative nitrogen fixation. This is why we investigated the diversity of potential nitrogen-fixing bacteria in the rhizosphere of white lupin. As a leguminous plant, white lupin also forms nodules where symbiotic nitrogen fixation with bradyrhizobiae takes place and usually, these nodules do not form a the same locations as cluster roots. We hypothesized that in addition to the symbiotic fixation in non cluster roots, associative nitrogen fixation by free-living rhizobacteria might take place in cluster roots. We used the *nifH* gene (coding for the dinitrogenase reductase) as a marker of potential nitrogen fixation and performed a RFLP analysis, as well as a cloning-sequencing approach. Positive amplification of the *nifH* gene from the DNA extracted from the root fraction was obtained for non cluster roots, juvenile and senescent cluster roots. No amplification was observed for mature cluster roots, which suggests that associative nitrogen fixation is not taking place, at least not to a detectable level, at this stage, despite the high availability of organic carbon. We also performed PCR amplification of the *nifH* gene on cDNA reverse-transcribed from extracted RNA. *NifH* mRNA amplification was positive for non cluster roots and, in one of four replicates, for juvenile and senescent cluster roots, indicating that associative nitrogen fixation might indeed occur in cluster roots. The restriction analysis (RFLP) revealed differences between the patterns of potential nitrogen-fixers associated with cluster roots and non cluster roots, as well as between the patterns associated with juvenile and senescent cluster roots. Since no report up to now investigated the communities of potential associative nitrogen fixers in the rhizosphere of white lupin, we applied a cloning-sequencing approach to determine to which species our pools of amplified *nifH* genes corresponded. The sequences retrieved from cluster roots shared a high similarity with known *nifH* sequences affiliated to *Bradyrhizobium* sp., whereas sequences coming from non cluster roots were surprisingly more distantly related to *Bradyrhiozbiae* and more closely to *Vibrio* or *Methylococcus* species.

The last experiment we performed on the theme of plant microbe interactions was dealing with the impact of phenolics on the rhizosphere bacterial communities. After analyzing in detail the secretion of isoflavonoids by white lupin's cluster roots, as well as their possible impact on bacterial and fungal growth, we were interested in the role of these compounds as potential selective or elective agents involved in the structuring of rhizosphere bacterial communities. Since white lupin cannot be genetically transformed, we used *Arabidopsis thaliana* to assess this question with flavonoid-deficient mutants. The *tt4* mutant no longer possesses a functional chalcone-synthase and is thus lacking flavonoids. We used a PCR-DGGE approach based on a fragment of the 16S rRNA gene to compare the bacterial communities associated with *tt4* and the corresponding wild-type plants. Both the present communities (DNA based DGGE profiles) and the active communities (RNA based DGGE profiles) were analyzed. As expected and previously observed in the case of white lupin, a lower richness of present communities was found in root-associated communities than in rhizosphere soil communities, which can be related to the selective effect of roots. The richness of bacterial communities was significantly influenced by the plant genotype and surprisingly, a lower richness was found for present communities in the *tt4* rhizosphere. The correspondence analysis revealed that the major factors accounting for the differences in community structure were i) the root proximity (rhizosphere soil *vs.* root), ii) the type of targeted community (present *vs.* active) and iii) the plant genotype (*tt4 vs.* wt). Our results clearly showed an effect of the plant genotype on the richness and structure of bacterial communities. Since the chalcone synthase is the only genetic difference between the *tt4* and the wild-type plants, one would be tempted to conclude that flavonoids are responsible for the changes observed in the structure of bacterial communities. However, pleiotropic effects of the mutation cannot be excluded, nor can we be sure that the lack of flavonoids does not induce other phenotypic effects, which are only indirectly related the flavonoid deficiency.

In this chapter, we investigated many aspects of rhizosphere bacterial communities and plant microbe interactions, with a strong focus on white lupin, as our plant of choice for the study of tolerance to phosphate deficiency. To overcome the research limitations that are imposed when working with a model plant which cannot be genetically transformed,

we resorted to *Arabidopsis* to be able to investigate the impact of phenolics on the structuring of bacterial communities, by comparing the rhizosphere communities associated with a flavonoid-deficient mutant and with the corresponding wild-type.

3.6.2. Conclusions and outlook

Despite the recognized role of microorganisms in nutrient cycling and plant nutrition, physiological studies on plants which are able to cope with nutrient deficiencies up to now rarely included the microflora component. Throughout the experiments described in this chapter, we have been able to fill, at least partly, this gap of knowledge that characterized white lupin or any other cluster-rooted species. Working on plant microbe interactions in the rhizosphere of white lupin and *Arabidopsis thaliana* bore the great advantage of knowing a lot about the plant and thus being able to use the available knowledge as a tool for the interpretation of the results obtained for the associated microbial communities. With a combined approach using both classical cultural methods and molecular tools, we were able to bring elements of answers to questions such as "is there a cluster-root specific microflora?", "how do the bacterial communities adapt to the changing conditions associated with cluster root development?", "are cluster root associated bacteria helping the plant through plant-growth promoting activities?" or, on the opposite "can the plant protect the secreted organic anions from microbial degradation?".

However, there are still many points which deserve attention and should be investigated in future studies. The most striking question obviously is "who are these cluster root-associated bacteria?" Even if a recent - and first - report investigated the phylogenic diversity of phytate hydrolyzing bacteria in the rhizosphere of white lupin (Unno et al., 2005) and identified the genus *Burkolderia* as a major genus among these isolates, this study did not take cluster roots into account and only performed the cloning and sequencing step on isolated strains. This constitutes a first interesting indication that bacteria belonging to the genus *Burkholderia* might be involved in the phosphate acquisition from organic pools of phosphate and more generally, might play a role in

white lupin rhizosphere. Bacteria belonging to the "*Burkholderia cepacia* complex" have been often found in the rhizosphere of plants (Coenye and Vandamme, 2003), and even if some were found to be plant pathogens (Parke and Gurian-Sherman, 2001), most of them were shown to promote plant growth in many different ways, among which nitrogen fixation and even nodule formation (Coenye and Vandamme, 2003), siderophore production (Bevivino et al., 1998), phosphate acquisition (Unno et al., 2005) and antagonistic properties against pathogenic fungi, from Oomycetes (Heungens and Parke, 2000) to Basidiomycetes (Kang et al., 1998). In addition, bacteria belonging to the *Burkholderia cepacia* complex have been shown to produce N acylhomoserine lactones (Gotschlich et al., 2001), signal molecules involved in cell to cell communication and quorum sensing processes, which play an important role in rhizosphere competence and root colonization (Zhang and Pierson, 2001). However, before the relative importance and the plant growth promoting impact of this genus in white lupin rhizosphere can be really demonstrated, a cloning-sequencing approach should be used without the bias introduced by the cultivation step and a precise sampling procedure should be performed, allowing separation of at least cluster and non cluster roots.

Our results on potential associative nitrogen fixation in the rhizosphere of cluster roots also would require further investigations: *nifH* gene could be amplified from cluster root samples, and at the level of the mRNA in some cases, indicating a potential nitrogen fixation around cluster roots. This should be repeated and, with a more adapted extraction procedure and RT-PCR setup, the amplification of mRNAs from cluster roots should become reproducible. Clearly, a measurement of nitrogen fixation on whole cluster roots, as well as on the different stages separately, would be the logical following of this study and would bring the final piece of evidence needed to establish the presence or absence of nitrogen fixation in cluster roots.

The question of bacterial auxin production and its role in cluster root formation should also be investigated more thoroughly: we observed that the auxin producing strains were more frequent in the rhizosphere of young cluster roots. It would be interesting to analyze bacterial auxin production around root zones where cluster roots will emerge later, since

auxin is needed at the very beginning of cluster root initiation, even prior to cluster root emergence, but for obvious reasons, this is a very difficult thing to do, since we do not know in advance in which zones of the root system cluster roots will initiate.

Finally, the role of bacteria in the formation of cluster roots is still a non resolved issue. Such an impact of bacterial impact on cluster root formation has been postulated long ago and some studies have been conducted to investigate the question (Gardner et al., 1982a; Lamont and McComb, 1974; Malajcuk and Bowen, 1974). In these experiments, conclusions were quite controversial, some of them showed the formation of cluster roots, while others did not, or at least showed a much higher number of cluster roots formed when bacteria were present. In most cases, negative controls consisted of autoclaved soil, which is no ideal control, since autoclaving alters the soil structure and can liberate toxic substances from the soil. In this work, as well as in the preceding diploma work, we tried several ways to set up a system where both non sterile and sterile plants could be grown in favorable conditions, so that they would reach the age where cluster root formation starts (about 21 days). Unfortunately, we never got any cluster roots in the plants grown in sterile or non sterile conditions and were thus unable to elucidate the question of the bacterial impact in cluster root formation. A particular challenge is to maintain the aerial parts of the plants in a sterile environment. A growth of plants in hydroponic culture under a sterile hood would represent a possible solution to this problem, provided the ventilation is not drying the air too much. One must admit, though, that the probability that cluster root initiation depends on bacteria is weakened by the fact the white lupins "always" produce cluster roots when grown in P deficiency in hydroponic cultures, where no particular inoculation of lupin-adapted bacteria is performed. This would mean that any bacterium "passing by" would be sufficient to allow formation of cluster roots and this is to our opinion not a reasonable assumption. However, it would be satisfactory to be able to grow white lupins in sterile and yet plant-friendly conditions and see if P deficiency causes cluster root formation in the absence of bacteria also, so we would able to put a final answer to this long standing question of the potential role of bacteria in cluster root formation.

CHAPTER IV

Synthesis And Conclusions

4.1. SYNTHESIS

Throughout the experiments conducted in this Ph.D. thesis, we were able to gain additional knowledge on the mechanisms which enable white lupin to grow on soils with sparingly available phosphate. We investigated cluster root secretion physiology and phosphate acquisition, as well as plant microbe interactions in the rhizosphere of *Lupinus albus* and *Arabidopsis thaliana*.

New roles for the enzyme ATP citrate lyase were proposed, namely i) the regulation of the organic acid preferentially secreted by cluster roots and ii) the supply of acetyl CoA for the flavonoid biosynthetic pathway. Isoflavonoid contents and secretion patterns were characterized in growing cluster roots of white lupin. Genistein and hydroxygenistein, as well as their glycosylated conjugates, were the major isoflavonoids identified. We could show that while internal contents remained stable during cluster root development, the amounts secreted varied in function of cluster root stage. Highest secretion was observed at the juvenile and immature stages of cluster roots. The secretion of isoflavonoids by white lupin seedlings was strongly modified when elicited with the presence of bacteria or fungi and the response was strain-specific, some strains decreasing secretion, some other increasing it and a few strains showing no effect at all.

The secretion by cluster roots of high amounts of organic acids, protons and isoflavonoids was likely to have a strong impact on the rhizosphere microbial communities and we assessed this question as well. Our results showed that the bacterial communities were influenced by the secretion activity of cluster roots: a reduced bacterial abundance (cultivable and total), as well as a decreased richness (DGGE profiles) characterized the mature stage of cluster roots. Approximately 50 % of the bacterial populations isolated from the mature stage were able to grow in a low pH (4) medium, while only 20 % of the juvenile isolated bacteria could do the same, suggesting that the pH decrease occurring transiently in the rhizosphere of mature cluster roots could be the cause of the decreased abundance of bacteria

observed a this stage. In contrast, no inhibitory effect of the secreted isoflavonoids could be evidenced *in vitro* on bacteria. As for fungi, we did not see any inhibition of the vegetative growth (mycelium), but we observed an enhanced sporulation in several fungi, both for isolates and collection strains. We used a *Fusarium oxysporum* strain to study this phenomenon in more details and with a TLC plate based bioassay, we could observe that sporulation co-localized with the isoflavonoid (Gibbs reagent) which showed highest UV absorption and was thus thought to be the major peak in the HPLC profile, genistein 6"-*O*-malonyl-O-glucoside. When further purifying the fraction, this sporulation effect could not be reproduced, which indicates that more than one compound could be involved in this phenomenon and that they might act in a synergistic way. In addition to the release of organic acids and isoflavonoids into the rhizosphere, white lupin cluster roots also secreted antifungal enzymes and the activity of both chitinases and glucanases were highest at the immature stage, the stage preceding citrate secretion. Taken all together, these results indicated that white lupin developed a complex strategy to limit bacterial and fungal degradation of the phosphate-solubilizing citrate: a pH decrease to transiently limit bacterial growth and a burst of phenolics enhancing fungal sporulation, as well as secretion of antifungal enzymes to lower fungal breakdown of citrate.

However, our results also indicated that the rhizosphere microflora displayed plant growth promoting properties and bacteria cannot thus be considered simply as "the bad citrate degraders". We observed that proportions of auxin producing bacteria were highest in the rhizosphere of juvenile cluster roots, suggesting that bacteria might contribute to the auxin needed for cluster root initiation. Moreover, our study on *nifH* diversity in the rhizosphere of white lupin indicated that associative nitrogen fixation might take place in the rhizosphere of juvenile and senescent cluster roots, in addition to the symbiotic fixation in nodules forming on non cluster roots.

4.2. CONCLUDING REMARKS

The carrying out of this interdisciplinary work was possible thanks to the collaboration between plant physiologists, microbiologists and chemists. The integration of different fields and approaches around a biological question such as a plant P acquisition strategy has given us the opportunity to analyze the subject from different perspectives, which is a necessity when studying rhizosphere processes or interactions between organisms. Moreover, this interdisciplinary approach gave me the possibility to become familiar with a broad range of experimental techniques.

I believe that the general aim, the better understanding of cluster root secretion physiology and its impact on rhizosphere microbial communities, has been achieved and that many questions have found an answer, at least a partial one.

Of course, working with a model plant which cannot be genetically transformed limits the molecular tools available. However, other ways can be found to address the questions of interest, for instance the use of another model plant for which mutant lines are available. This is what we did when we investigated the impact of flavonoids on the structure of rhizosphere bacterial communities. Except for this aspect, white lupin offers many advantages as a model plant. The most important feature is the available knowledge on the different stages of cluster root development. We have shown that the investigated rhizosphere processes of root secretion and microbial responses shared the common feature of occurring in a transient and stage specific way. Thus, the understanding of i) P acquisition by white lupin, ii) cluster root secretion and iii) the interactions with the associated microflora would not have been possible if entire cluster roots only had been analyzed. The detailed characterization of cluster root development in white lupin enabled us to investigate in detail the processes of root secretion and plant microbe interactions in well-defined samples. In addition, white lupin can easily be grown in different experimental systems like hydroponics, rhizoboxes or microcosms and produces a high root biomass, an important feature when studying secondary

compounds like isoflavonoids, which are secreted in much lower amounts that organic acids for example. Last but no least, white lupin is a very promising crop for low input agriculture due to its ability to grow on soils with low available nitrogen and phosphate. This gives an additional interest to such a study as this Ph.D. thesis, since the acquired knowledge should be transferable to concrete field situations and hopefully could help improving the agricultural practices in soils with low available phosphate.

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ATP citrate lyase: cloning, heterologous expression and possible implication in root organic acid metabolism and excretion

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ABSTRACT

In order to cope with phosphate deficiency, white lupin produces bottle-brushed like roots, so-called cluster or proteoid roots which are specialized in malate and citrate excretion. Young, developing cluster roots mainly excrete malate whereas mature cluster roots mainly release citrate. Mature proteoid roots excrete four to six times more carboxylates compared with juvenile proteoid roots. Using a cDNA-amplified restriction fragment length polymorphism (AFLP) approach we identified a gene coding for a putative ATP-citrate lyase (ACL) up-regulated in young cluster roots. Cloning of the lupin ACL revealed that plant ACL is constituted by two polypeptides (ACLA and ACLB) encoded by two different genes. This contrasts with the animal ACL, constituted of one polypeptide which covers ACLA and ACLB. The ACL function of the two lupin gene products has been demonstrated by heterologous expression in yeast. Both subunits are required for ACL activity. In lupin cluster roots, our results suggest that ACL activity could be responsible for the switch between malate and citrate excretion in the different developmental stages of cluster roots. In primary roots of lupin and maize, ACL activity was positively correlated with malate exudation. These results show that ACL is implicated in root exudation of organic acids and hence plays a novel role in addition to lipid synthesis. Our results suggest that in addition to lipid biosynthesis, in plants, ACL is implicated in malate excretion.

Key-words: *Lupinus albus*; ATP-citrate lyase; cluster roots; citrate; malate; root excretion; root exudation.

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INTRODUCTION

Phosphorus (P), is one of the most limiting nutrients in natural and agricultural ecosystems, due to the low concentrations of free inorganic phosphate (P_i) in the soil solution, which is the sole form of phosphorus absorbed by plant roots (Marschner 1995). The strategies of most plants to overcome phosphate starvation are (i) the synthesis of high affinity transporters, which can absorb P_i even if the concentration in the soil solution is very low; (ii) modifications in root morphology and the association with mycorrhiza, mainly improving spatial acquisition of available phosphate; (iii) the secretion of phosphohydrolases which liberate P_i by hydrolysis of organic P forms; (iv) the acidification of the rhizosphere by enhanced net extrusion of protons for mobilization of acid-soluble P fractions in calcareous soils; and (v) the excretion of carboxylates as organic chelators, mobilizing sparingly soluble Fe, Al and Ca phosphates by complexation of the metal cations. Rhizosphere acidification is a widespread response to phosphorus deficiency, particularly in dicotyledonous plants. In contrast, excretion of considerable amounts of carboxylates effective in phosphate solubilization seems to be restricted to a limited number of plant species (Marschner 1995; Neumann & Römheld 1999, Neumann & Martinoia 2002). White lupin and members of the Proteaceae are able to excrete far larger amounts of carboxylates than any other plant species investigated so far (Dinkelaker *et al.* 1997; Neumann & Martinoia 2002). Compared with rhizosphere acidification, this strategy has the advantage that phosphate is not only liberated from acid-soluble P fractions, but also by anion exchange from Fe–Al–P complexes found in many acidic soils. In these plant species, exudation of organic acid anions occurs at well-defined parts of roots, where short, clustered lateral roots (proteoid or cluster roots), are formed. White lupin is the best-described system forming proteoid roots. Formation of proteoid roots follows a well-defined developmental pattern. Young, growing proteoid roots release mainly malate and only low amounts of citrate whereas immature proteoid roots excrete similar amounts of citrate and malate. In contrast mature proteoid roots excrete far higher amounts of carboxylates, mainly citrate

and strongly acidify the rhizosphere. This is associated with a shift from malate to citrate accumulation in the root tissue with increasing age of the clusters (Neumann *et al.* 1999, 2000). The high demand for carbon used for the synthesis of carboxylates is sustained by the up-regulation of several enzymes, i.e. PEPcarboxylase (Johnson *et al.* 1996a; Johnson, Vance & Allan 1996b), sucrose synthase, phosphoglucomutase and fructokinase which are involved in the glycolytic pathway (Massonneau *et al.* 2001).

Although up-regulation of these enzymatic activities can explain increased exudation of carboxylates from cluster roots in general, the reasons for the differential exudation of malate or citrate in different root zones (juvenile and mature clusters) still remain an open question. Using the cDNA-amplified restriction fragment length polymorphism (AFLP) technique, we identified a large number of genes with putative differential expression in young and mature proteoid roots. Among these genes, we also identified a putative ATP-dependent citrate lyase (ACL). ACL (EC 4.1.3.8) catalyses the formation of acetyl-CoA and oxaloacetate from citrate and CoA with a concomitant hydrolysis of ATP. Due to its instability, the study and cloning of ACL has been delayed and it was only in 1990 that the first animal ACL was cloned (Elshourbagy *et al.* 1990). Several reports on the plant ACL have been published and its activity has been found mainly in young growing parts and during seed maturation (Kaethner & ap Rees 1985; Ratledge, Bowater & Taylor 1997). In developing seeds of *Brassica napus*, Ratledge *et al.* (1997) found that most of the ACL activity was localized in chloroplasts and established a correlation between ACL activity and the rate of lipid synthesis. In other plants, cell fractionation studies showed that ACL was exclusively localized in the cytosol (Kaethner & ap Rees 1985; Ma *et al.* 2001) and a role in terpenoid synthesis in response to pathogens was postulated (Suh *et al.* 2001).

Despite the importance of ACL, to our knowledge, plant ACL has yet not been cloned. Molecular identification of ACL will provide the opportunity to learn more about the role of the corresponding gene product in plant metabolism. Indeed, as shown in this report, cloning of lupin ACL revealed that in contrast to animals, plant ACL is constituted by two different subunits and that in addition to its well-established role in lipid synthesis, it may also play a role in malate exudation in roots.

MATERIALS AND METHODS

Plant material

Growth of white lupin (*Lupinus albus* L. cv. Amiga; Süd-westdeutsche Saatzucht, Rastatt, Germany) in presence (+P) or absence (–P) of P source has been previously described (Massonneau *et al.* 2001). Maize (*Zea mais* L. cv. Delprim; Delley Semences et Plantes SA, Yverdon, Switzerland) was pre-germinated for 4 d and grown hydroponically in complete (+P) medium. Plants were grown at 22 °C and 65% relative humidity with a light period of 16 h at 200 μmol m^{-2} s^{-1}.

Harvest of different root parts

The different stages of proteoid roots were harvested as described by Massonneau *et al.* (2001). The apex (1 cm) of fast-growing secondary roots (named N2 in our previous studies) are harvested on secondary roots holding clusters. In order to differentiate the developmental stages of root clusters, the root system was immersed in a pH-indicator solution, which indicates acidification in mature cluster regions (Neumann *et al.* 1999). In experiments in which organic acid excretion was measured, root segments were washed twice for 1 min in the bath solution. Demonstration that organic acid contents measured in the bath solution corresponds to excretion and not to organic acid release through the cut surface has been described in Neumann *et al.* (1999) for proteoid roots and by Ryan, Delhaize & Randall (1995) for normal root tips.

Collection and analysis of exudates

Excised roots were rinsed in water and incubated for 1 h in water containing penicillin (Massonneau *et al.* 2001). Malate contents were determined using the Malate Test Kit (Boehringer, Mannheim, Germany).

Heterologous expression of the ACL in yeast

Saccharomyces cerevisiae W303 (MAT-a ade 2–1 can 1–100 hi 3–11 leu 2–3 trp 1–1 ura 3–1) were grown on appropriate selective media and transformed via the Li-acetate protocol (Lundblad 1997). *La ACLA* and *La ACLB* full-length cDNAs in pBluescript were excised with XhoI and NotI and ligated in the pNEV vectors (Sauer N. et Scholtz J. 1994) containing Ura, respectively, Leu as markers. This system allowed the constitutive expression by the pma-1 promoter (Villalba *et al.* 1992). Transformed yeast cells were grown to OD 4–5 in selective media and subsequently transferred to YPD medium for 1 h 30 min before preparing spheroplasts (Lundblad 1997).

Extraction of soluble proteins and ACL assay

Frozen plant tissues were ground in liquid nitrogen and homogenized with 3 vol. of extraction buffer (0·1 M Hepes-KOH pH 7·5, 5 mM $MgCl_2$, 2·5 mM dithiothreitol, 3 mM Na-DEDTC (diethyldithiocarbamate), 1 mM eethylenediaminetetraacetic acid, 1 mM benzamidine, 1 mM phenylmethanesulfonylfluoride, 3% polyvinylpolypyrrolidone K30).

Yeast spheroplasts were lysed osmotically and gently homogenized at 4 °C in 1·5 vol. of extraction buffer (complemented with Na-Citrate to a final concentration of 5 mM) using a syringe and needle. After centrifugation (25 min, 12 000 × *g*, 4 °C) the supernatant was rapidly used to determine the ACL activities and protein concentrations (DC Protein Assay kit; Bio-Rad).

ACL (EC 4·1.3·8) activity was determined spectrophotometrically at room temperature, using the malate dehydrogenase coupled assay. The assay mixture contained 0·2 M

Tris pH 8·4, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20 mM Na_3-Citrate, 0·2 mM Coenzyme A, 10 mM ATP (omitted in blanks), 0·2 mM NADH, 0·4 U mL^{-1} malate dehydrogenase. Values were taken after 30 min and 10 min for plants and yeast, respectively. These time points were chosen since prolonged incubation resulted in the inactivation of the enzyme. Blanks were performed by omitting ATP or Coenzyme A, resulting in similar values.

cDNA-AFLP

The RNAs from juvenile, mature and senescent cluster roots were compared using the cDNA-AFLP technique (Bachem *et al.* 1996). The procedure used in this work is described by Massonneau *et al.* (2001).

Cluster root library construction and screening

The library was constructed from mRNA isolated from juvenile cluster roots. cDNAs synthesis and cloning were conducted using the ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit (catalogue no. 200400 and catalogue no. 200450; Stratagene, Amsterdam, The Netherlands). The screening of *La ACLA* was performed using a polymerase chain reaction (PCR) approach. Primers were deduced from the sequence of the cDNA-AFLP clone showing homology with the C-terminal part of the rat ACL. Primers 5′GTGCCATTGATGATGCTGCT3′ and 5′TGTTTGCCTTCGTGAGAGTG3′ amplified a 249 bp fragment. Six independent clones have been completely sequenced and showed approximately the same size, the longest being 2222 bp.

To screen *La ACLB*, degenerated primers have been designed from consensus regions between cotton, alfalfa, soybean, tomato and *Arabidopsis thaliana*. 5′TGAGYTA GTARASARRGARCCNTGG3′ and 5′ACCICCICC NGCNACCATNGTCCA3′ amplified a fragment from which specific primers were deduced to screen the library. Six independent clones were isolated and completely sequenced. They showed approximately the same size, the longest was 1618 bp.

Reverse transcriptase-polymerase chain reaction

One million copies of pAW109 mRNA (Perkin Elmer, Applied Biosystems, Foster City, CA, USA) was mixed with 1 μg of DNA-free total RNA. Reverse transcriptase (RT)-PCR was performed as described by Massonneau *et al.* (2001) using (alpha)^{33}P-labelled dATP. One-hundredth of the RT reaction product was used to perform PCR. Two sets of primers were used in the same tube, one specific for pAW (AW112 and AW113, amplifying a 301 bp fragment) and one for *La ACLA* (5′GCTGGAGCTAAG AGTGGTGG3′ and 5′CTGTCACGAGCATCCTTGAA3′ amplifying a 600 bp fragment) or *La ACLB* (5′CGGGTC CATAAACCTCAATG3′ and 5′TGGTCGATGGAAAG CCTTAT3′ amplifying a 603 bp fragment). The annealing temperature was 60 °C. After 24 cycles, when strict proportionality between cycle number and amplification was observed for all tissues studied, the PCR fragments were separated on an agarose gel and blotted onto a nylon membrane for autoradiography. An additional control was performed using the ribosomal protein 60S as probe.

RESULTS

One of our interests is to understand the mechanisms leading to the exudation of the huge amounts of carboxylates by mature proteoid roots and how the cellular metabolism changes during the conversion of young, growing proteoid roots to mature proteoid roots. In order to identify stage-specific expression of mRNA we performed a cDNA-AFLP analysis (Bachem *et al.* 1996) with juvenile, mature and senescent root clusters. In a former contribution we showed that several enzymes implicated in glycolysis, namely fructokinase, sucrose synthase and phosphoglucomutase were up-regulated in juvenile and mature cluster roots (Massonneau *et al.* 2001). Among the genes identified to be differentially expressed, we also identified a putative ATP-dependent citrate lyase. ACL catalyses the reaction which forms acetyl-CoA and oxaloacetate from citrate, CoA and ATP.

The length of the putative ACL clone was 278 bp and exhibited 71% similarity with the rat ACL over a stretch of 92 amino acids. Transcript analysis using semi-quantitative RT-PCR confirmed that the sequence identified was indeed highly expressed in juvenile proteoid roots and down-regulated in mature proteoid roots (see below).

Since, to our knowledge, no plant ACL has been cloned and functionally identified, we were interested to verify whether the cDNA-AFLP fragment corresponds to the ACL gene. PCR-based screening, using primers deduced from the putative ACL, of a lupin library made from juvenile proteoid roots, resulted in six independent clones, the longest being 2222 bp. Alignment of the lupin clone with the animal ACL (3303 bp) revealed that our clone was highly similar to the C-terminal half of the rat ACL. Comparison of the lupin sequence with the *Arabidopsis* database revealed two genes of similar size also corresponding to the C-terminal part of rat ACL (Table 1). Since these clones were nearly 1500 bases shorter than the full-length cDNA reported for rat, we performed a Northern blot. The size of the transcripts hybridizing with our probe was 2·3 kb, a size similar to the cDNA cloned for the lupin (not shown). A similarity search using the rat ACL gene revealed three putative *Arabidopsis* genes exhibiting a high homology to the N-terminal part of the rat ACL. These putative genes have no overlap with the genes coding for the C-terminal part mentioned above. ESTs from other plants corresponding to the N-terminal part of ACL are also present in the database. Using degenerated oligonucleotides corresponding to conserved parts of this putative subunit of ACL, we screened the lupin library and identified several clones of approximately 1600 bp. An alignment

Table 1. Comparison of *Lupinus albus* ATP citrate lyase (ACL) with ACL of *Rattus norvegicus* and putative ACLs of *Arabiopsis thaliana*

	L. albus		*R. norvegicus*			*A. thaliana*			
	Gene	Size (aa)	Gene	Size (aa)	(% similarity/ identity)	Gene	Size (aa)	(% similarity/ identity)	Chromosome
ACLA	AJ344108	423	P16638	1100	61/45	At1g60810	423	94/89	1
						At1g09430	424	91/81	1
						At1g10670	423	94/89	1
ACLB	AJ344107	608	P16638	1100	68/52	At3g06650	608	97/93	3
						At5g49460	608	97/93	5

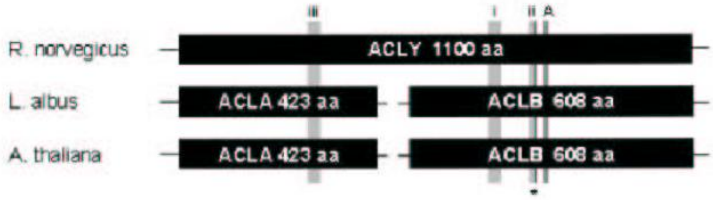

Figure 1. Subunit organization of *Lupinus albus*, *Arabidopsis thaliana* and *Rattus norvegicus* ATP citrate lyases. i, ii and iii are the ATP-dependent citrate/succinate-CoA ligase signatures, * indicates the phosphorylation sites corresponding to the active site observed in animal ACLs and A indicates the putative coenzyme A binding site.

of the lupin cDNA with the *Arabidopsis* and rat genes shows that the deduced gene products are highly similar (Table 1). As we supposed that in plants the ACL activity is dependent on two gene products, we called the N-terminal ACLA and the C-terminal part ACLB (Table 1, Fig. 1). As in their animal counterparts, we have identified within the subunit A the putative CoA binding domain and a conserved histidine, which is phosphorylated during the enzymatic reaction, two ATP-dependent citrate/succinate-CoA ligase signatures in this subunit and one in subunit B (Fig. 1). Interestingly, both in fungi and bacteria, ACL exhibit a similar organization pattern as in plants (Nowrousian *et al.* 2000; Kanao *et al.* 2001). In comparison with the animal ACL, *Chlorobium limicola* and *Lupinus albus* miss a region of about 60 amino acids located between subunit B and A in animal ACLs.

In order to prove that the two genes identified indeed code for the plant ACL, we performed expression studies. In a first attempt, we expressed the two gene products in *Escherichia coli*. On a sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel, we observed high levels of the corresponding proteins, however, no activity could be detected, most probably due to the fact that the ACL gene products were only present in inclusion bodies and hence insoluble. Therefore we decided to use yeast as expression system. The genome of *Saccharomyces cerevisae* does not contain genes corresponding to the ACL and in fact no ACL activity could be detected in wild-type yeasts (Fig. 2). In contrast, when the two ACL subunits were expressed in yeast under the control of a constitutive promoter, ACL activity could be detected. Expression of the A or B subunit alone resulted in negligible ACL activities, demonstrating that both subunits are required for ACL activity.

After the demonstration that the heterologously expressed clones code for the ACL, we were interested to correlate the expression of ACL with the enzymatic activity of its gene product in the different root types and to investigate whether ACL influences carboxylate exudation. Both subunits showed a very similar expression pattern, exhibiting high transcript levels in young, growing parts of roots and very low in senescent roots (Fig. 3). Quantification using a phosphoimager confirmed that the expression patterns of both subunits approximately paralleled the ACL activity in the different root types (Fig. 4b). The slight differences from one experiment to the other, mainly observed between juvenile and immature cluster roots, could be due to the difficulty in clearly separating both

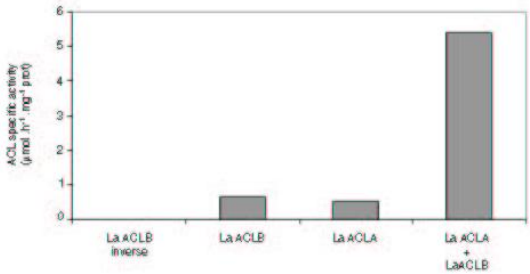

Figure 2. ATP citrate lyase activities measured in yeasts expressing the different lupin ACL subunits. The experiment was repeated four times, giving always the same pattern. A typical result is presented. Since unspecific NADH oxidation occurred and led to negative values when both subunits were not expressed together, the results are corrected taking the values obtained for yeasts containing the *La aclb* antisense construct as reference.

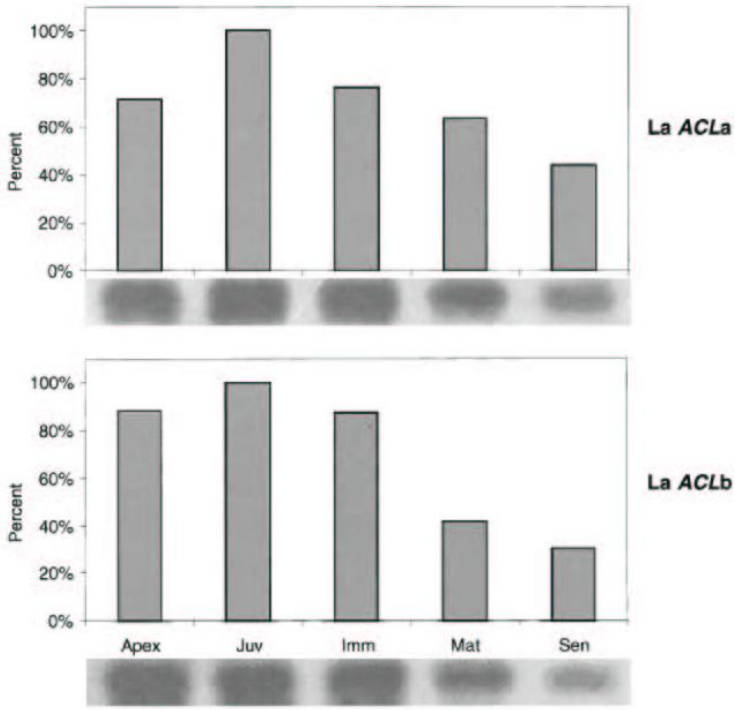

Figure 3. Transcript levels of the two subunits of ATP citrate lyase in different parts of lupin roots. Semi-quantitative RT-PCR was performed as described in 'Materials and Methods'. Quantification occurred by phosphoimager and is given in relative values (100% juvenile proteoid roots corresponding to the highest transcript levels. A control with total RNA stained with ethidium bromide is shown.

tissues. ACL activity was highest in juvenile proteoid roots and decreased in immature, mature and senescent proteoid roots. Extracts of root apexes exhibited ACL activities comparable with those of immature proteoid roots. Since our results suggest that ACL activity is highest in young, growing tissues, we measured this activity in lupin leaves. Young leaves showed a similar activity to root apex, whereas the ACL activity of adult leaves was of the same order of magnitude as senescent proteoid roots (not shown). This result confirms former observations, that ACL activity is mainly present in young tissues (Kaethner & ap Rees 1985; Ratledge *et al.* 1997).

Cluster roots are specialized in excreting high amounts of carboxylates and a peak is observed for mature cluster roots (Massonneau *et al.* 2001). Comparing the different stages of proteoid roots we observed that similar amounts of malate were excreted in juvenile, immature and mature proteoid roots (Fig. 4a). In contrast, a strong increase in citrate release can be observed during proteoid root development. The ratio of excreted malate to citrate was by far the highest in juvenile roots, which exhibit the highest ACL activity (Fig. 4c). Our results indicated that ACL could be implicated in malate excretion.

We have therefore used a second experimental system to test our hypothesis. In this case we have used primary roots of lupins (not containing proteoid roots) and maize. The first 30 mm of the roots were cut in four sections, two apical sections of 5 mm and the next two sections of 10 mm. In lupin as well as in maize the largest amounts of malate are excreted in the 5–10 mm section. This corresponds also to the part of the root exhibiting the highest ACL activity and has been shown to be an actively growing zone under similar culture condition in maize (Peters & Felle 1999). Root sections behind the 5–10 mm section excrete reduced amounts of malate and exhibit lower ACL activities. This result indeed suggests that ACL could be implicated in malate excretion (Fig. 5).

A detailed correlation analysis between ACL activity and malate exudation gives further confirmation of this hypothesis (Fig. 6). The calculation results in a highly significant regression coefficient ($r^2 = 0{\cdot}729$) and P-value (0·0001 calculated on 15–2-values). The statistical approach demonstrates a very good correlation between ACL activity and malate exudation under the conditions used and in the range of values of ACL activity and malate exudation measured. It can therefore be concluded that increased ACL activity and malate exudation are highly correlated.

DISCUSSION

Root exudation of organic acids plays an important role in a large number of processes, such as deficiency of mineral nutrients, particularly phosphate, exposure to toxic metals, such as aluminium or lead and to hypoxia (Neumann & Römheld 1999; Ryan & Delhaize 2001; Neumann & Martinoia 2002). In addition, organic acid exudation can also selectively stimulate microbial activities in the rhizosphere with impact on the availability of nutrients. At least in some

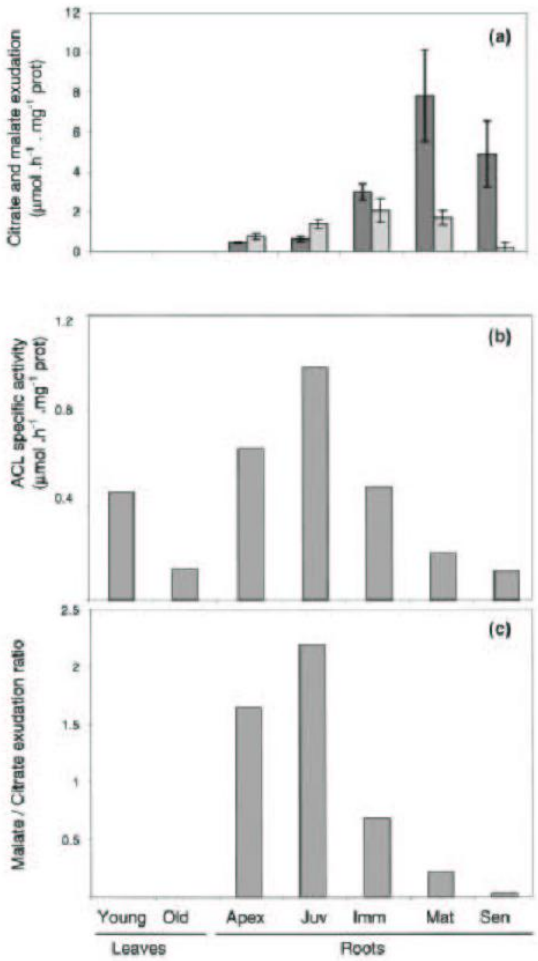

Figure 4. (a) Malate (light grey) and citrate (dark grey) excreted by different types of proteoid roots (results from a previous work by Massonneau *et al.* 2001). (b) ATP citrate lyase *in vitro* activities measured in leaves and in different root types of white lupin. The experiments were repeated at least four times, and the distribution pattern of enzymatic activities obtained in different root zones was highly reproducible. Due to the variations of the absolute enzyme activities, one single, typical experiment is shown. Measurements were performed in triplicate, SD are not given since too small to be visualized. (c) Ratio of malate to citrate excreted in different root types.

cases, exudation of carboxylates implicates the activation of a carboxylate channel (Kollmeier *et al.* 2001) and changes in cellular metabolism. In lupin, proteoid roots formed during phosphate starvation exhibit increased PEP carboxylase and citrate synthase activity and concomitant decreased aconitase activity (Neumann *et al.* 1999). In addition, increased activity of glycolytic enzymes suggests a high carbohydrates demand for biosynthesis of carboxylates (Massonneau *et al.* 2001). During the development of proteoid roots the pattern of exuded organic acids changes. In young, developing proteoid roots malate is the major carboxylic acid excreted. In contrast, mature proteoid roots excrete mainly citrate, which is more efficient in desorbing phosphate from Al–Fe–P complexes. In a cDNA-AFLP approach we have identified a cDNA coding for a putative ACL highly expressed in juvenile proteoid roots. Since the products of the ACL reaction are acetyl-CoA and oxaloacetate, which is readily reduced to malate, we have investigated the role of ACL in organic acid exudation.

Cloning of the full-length cDNA coding for the putative ACL revealed that in plants the ACL was not constituted by a single but by two different polypeptides corresponding to the N- and C-terminus of the animal ACL. A similar structure of the ACL has been postulated for fungi. However, the functional activity of these two subunits still remained to be proven. A very recent publication demonstrated that *C. limicola* exhibits a similar structure and that both subunits are required for ACL function. Our results demonstrate that this is also true for plants.

ACL activity measurements paralleled the expression pattern observed for both genes coding for the ACL, indicating that the transcript levels reflect the activity of the enzyme and that the enzyme is probably regulated at the transcriptional level. The fact that both subunits follow the same expression pattern implies a common regulation. However, the fact that ACL is encoded by two genes (*ACLA* and *ACLB*), may indicate that in plants additional regulatory mechanisms are involved compared with animals.

ACL activity has been extensively studied in animals and it has been shown to be a key step for lipid biosynthesis providing acetyl-CoA. A similar function has been postulated in rape seeds (Ratledge *et al.* 1997). In this case, ACL activity was localized in plastids, and the activity was correlated with lipid accumulation. In contrast, Kaethner & ap Rees (1985) localized pea ACL in the cytosol. A hint that ACL may be localized differentially according to the plant species was given by Rangasamy & Ratledge (2000), who found ACL activity predominantly in chloroplasts in rape and spinach, whereas in pea and tobacco, distribution was mainly cytosolic. The sequences of the lupin and *Arabidopsis* genes have no obvious transit peptide (search with PSORT, version 6·4) indicating that at least in these plants, ACL could be localized in the cytosol. Preliminary results using a GFP fusion protein also suggest a cytoplasmic localization. As membranes are considered to be impermeable to acetyl-CoA, it has to be used at its production place in the cytosol, for example, for synthesis of cytosolic terpenoids such as sterols or synthesis of malonylCoA used for flavonoid or ACC production, or transported to another site via an acetyl-CoA translocator. An acetyl-CoA translocator has already been cloned in animals (Kanamori *et al.* 1997; Bora, Kanamori & Hirabayashi 1999). However, an acetyl-CoA transporter has not been identified so far in plants and no homologue of animal transporters can be found in plant databases.

In lupin and maize, the highest ACL activities were found in growing tissues such as young leaves and elongation

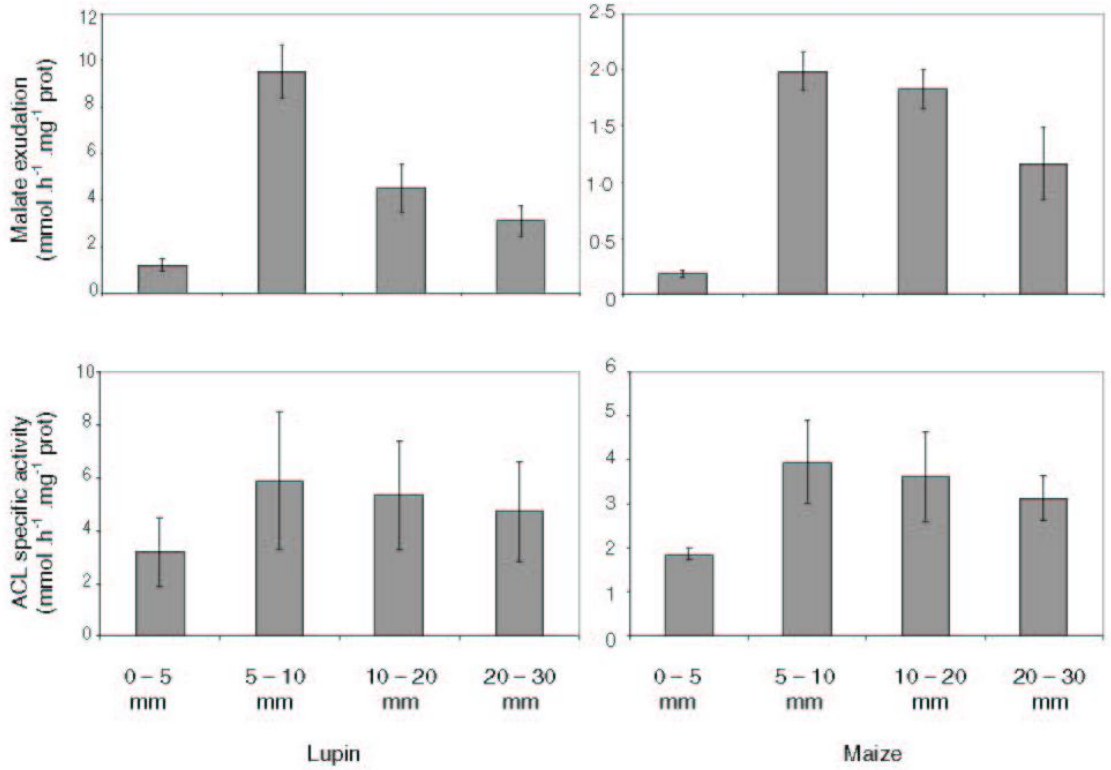

Figure 5. Malate exudation and ATP citrate lyase activities in different root zones of maize and white lupin. Root zones are indicated using the primary root apex as origin. Data represent the mean ± SD of three (lupin) or four (maize) independent experiments, each with three replicates.

zones of roots. In maize, the root elongation rate is maximal 6 d after germination and the elongation zone was identified between 3 and 8 mm from the apex (Muller, Stosser & Tardieu 1998). The correlation of the highest ACL activities with the elongation zone suggests that one role of ACL may indeed be linked to lipid biosynthesis.

Investigations of ACL function have focused on the acetyl-CoA production and lipid biosynthesis. In contrast, little attention has been paid to the second compound produced during this reaction, oxaloacetate, which is readily reduced to malate. This is probably due to the fact that in animals the role of malate is not as complex as in plants (Martinoia & Rentsch 1994). In lupin proteoid roots PEP-Case is highly expressed and thus constitutes a second pathway to produce malate (Fig. 7). Part of malate will be used for respiration, while the excess can be stored within the vacuole (Martinoia & Rentsch 1994). This strategy has been demonstrated mainly for leaves. In roots, both vacuolar storage as well as excretion have often been observed (Ryan & Delhaize 2001). Our results on primary roots of lupin and maize show that root parts exhibiting a high ACL activity excrete more malate. This may be due to the fact that in fast-growing zones a high demand for acetylCoA exists and that not all malate is used for respiration. A slightly different situation is present in proteoid roots, which have a specialized metabolism allowing them to excrete large amounts of carboxylates. In young, growing cluster roots the main organic acid excreted is malate. In a later fully developed stage, excretion is strongly increased

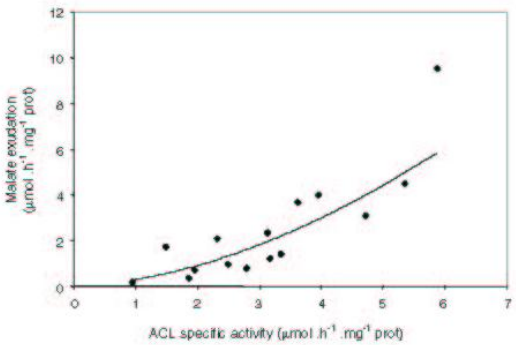

Figure 6. Correlation of ATP citrate lyase activity and malate exudation measured in maize and lupin roots. Data are means of three or four independent replicates. The curve $y = 0{\cdot}2543x^{1{\cdot}7646}$ fits the values ($r^2 = 0{\cdot}729$ and P-value = 0·0001).

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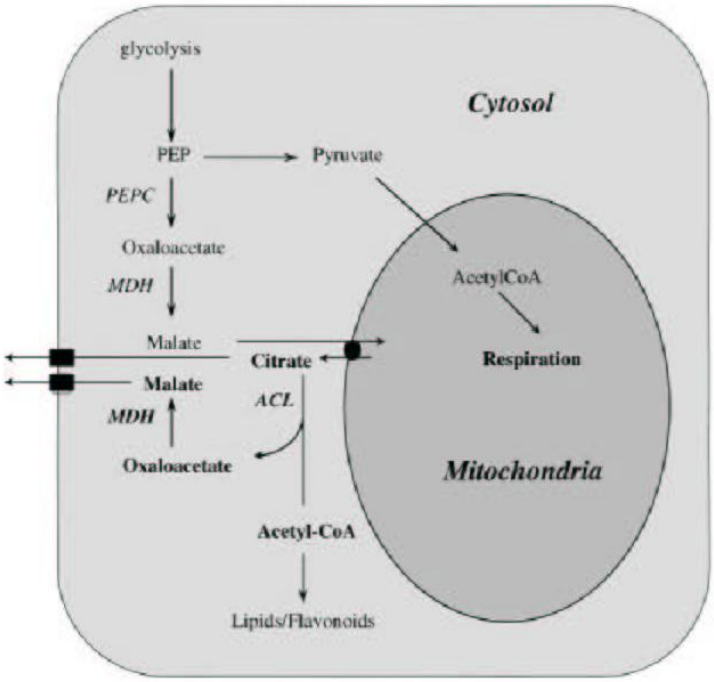

Figure 7, Schematic drawing of the metabolic activities implicated in malate release in juvenile proteoid roots.

and the main carboxylate released is citrate. This switch between malate and citrate excretion is illustrated by the change in the exuded malate/citrate ratio. A good correlation can be found between ACL activity and the ratio between the two organic acids, indicating that in proteoid roots, ACL plays a role as a metabolic switch. In mature proteoid roots ACL activity is decreased and the citrate removed from the Krebs cycle is no longer readily converted to malate. The cells produce large amounts of citrate, which cannot be used in metabolic pathways and are therefore forced to excrete citrate to maintain cellular homeostasis. This is reflected by a shift from preferential malate production in juvenile clusters to almost exclusive citrate production in mature and senescent clusters (Neumann *et al.* 1999, 2000). ACL activity, which links malate and citrate (Fig. 7), is therefore likely to be responsible for the switch in the organic acid excreted. The reduced ACL activity in mature proteoid roots favours the production and the release of citrate.

This may have important consequences from the ecological point of view. Consulting the complex formation constants with metals or mobilization of rock phosphate shows that citrate is much more efficient than malate (Ryan & Delhaize 2001). Exudation of citrate instead of malate may therefore improve P acquisition and the ability to cope with adverse soil chemical conditions such as Al toxicity. However, a mechanism of detoxification to avoid over-accumulation of organic acids in the root tissue may be regarded as the original function of intense root exudation of malate and citrate.

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