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Secretion activity of white lupin's cluster roots influences bacterial abundance, function and community structure

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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 excretion 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 excretion 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 excretes 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 excretion 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.

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 excretion 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 excretion 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

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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 excreted, 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 excretion physiology (Gardner et al., 1982a,b, 1983; Johnson et al., 1994; 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 excretion 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 excreted: at the juvenile stage, cluster roots exude mainly malate and do not acidify the soil. When cluster roots reach maturity, they excrete much higher amounts of carboxylates, mostly citrate. At this stage, a strong acidification of the soil is observed. However, carboxylate excretion and acidification are biochemically independent processes. Carboxylates are excreted 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 excreted. The excretion 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 micro-organisms. Preliminary results have shown that the excretion pattern of phenolics also changes during cluster root growth, with the highest excretion 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 con-

sumers (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 to assess the shifts in total (active and non active) and active communities that occur as the root grows. Over this time scale, 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.

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 2 h 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 × 30 × 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

In order to characterize micro-organisms 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 1A) 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 min, stages were revealed as shown in Figure 1B: 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 1 g 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%, 100 μl 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^{TM} 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.

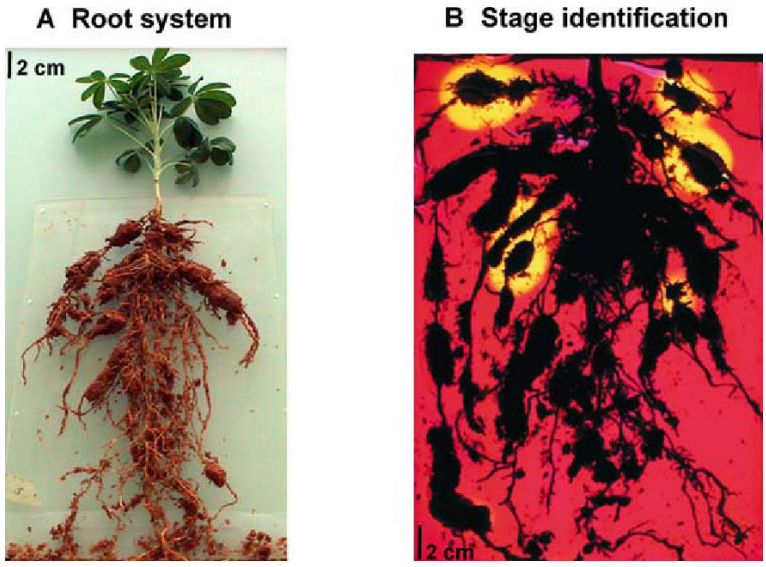

Figure 1. Harvest of the root system and identification of cluster root stages. A. Root system with adhering rhizosphere soil around cluster and non cluster roots. B. Revelation of acidification at the mature stage of cluster roots. The root system was overlaid by an agar-gel containing a pH indicator. After 20–30 min, yellow zones appeared, revealing the mature stage of the cluster roots. See material and methods for more details.

DNA extraction

DNA extraction was performed using a bead beating technique (FP120 FastPrep^{TM} 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^{TM}matrix D tubes (Bio101) and RNeasy® Plant Kit (Qiagen). 450 μL of RLT Buffer (Qiagen) were added to each FastRNA^{TM} tube containing the samples. The tubes were shaken twice for 10 s at 6 ms^{-1} using a FastPrep^{TM} 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^{TM} 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 × ImProm-II^{TM} Reaction Buffer, 1 U/20 μL RNasin, 6.0 mM $MgCl_2$, 0.5 mM of each dNTP, 5% (vol/vol) ImProm-II^{TM} 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 re-

verse 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) 1× 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) acrylbisacrylamide (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 1× 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 1× 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).

Results

Characterization of bacterial abundance associated with the different root stages

Bacterial abundance transiently decreased at the mature stage of cluster roots (Figure 2), 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 excretion 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.

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 1). 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 1. 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 1). Overall, these frequencies were quite high (more than 50% of the tested strains).

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

From the DGGE profiles obtained (Figure 3), 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 (4A and 4B). 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. Figures 4A and 4B show the selective effect of roots on the community DNA profiles: a decrease of the richness (number of bands, Figure 4A) and of the diversity (Shannon index, Figure 4B) 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 effect of root exudates on the microflora. Figure 4C 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 excretion. 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 5). The result of the global correspondence analysis on all the samples is presented in Figure 5A. 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 5B), 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 5C) was more influenced by root type and cluster root age than the structure of present communities (Figure 5B). A further interesting point in active communities (Figure 5C) was that the bacterial communities isolated from the root fraction of the mature samples (empty triangles) were well separated from

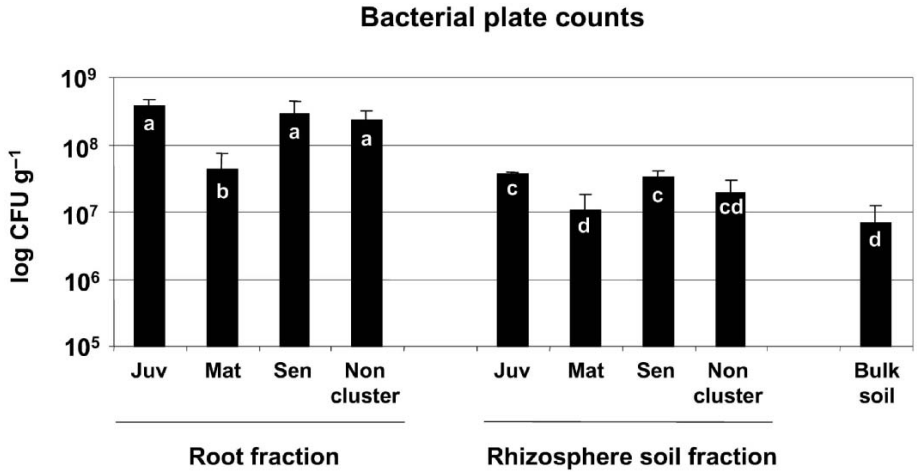

Figure 2. Numbers of CFU (colony forming units) g^{-1} dry weight in function of root stage and type, for the root fraction and the rhizosphere and bulk soil fractions. Juv: juvenile, Mat: mature, Sen: senescent. CFU were counted on the non-selective Angle medium. Values with different letters (a, b; 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).

Table 1. Frequencies (in % of total isolated bacteria) of bacteria 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 pooled 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}

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.

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

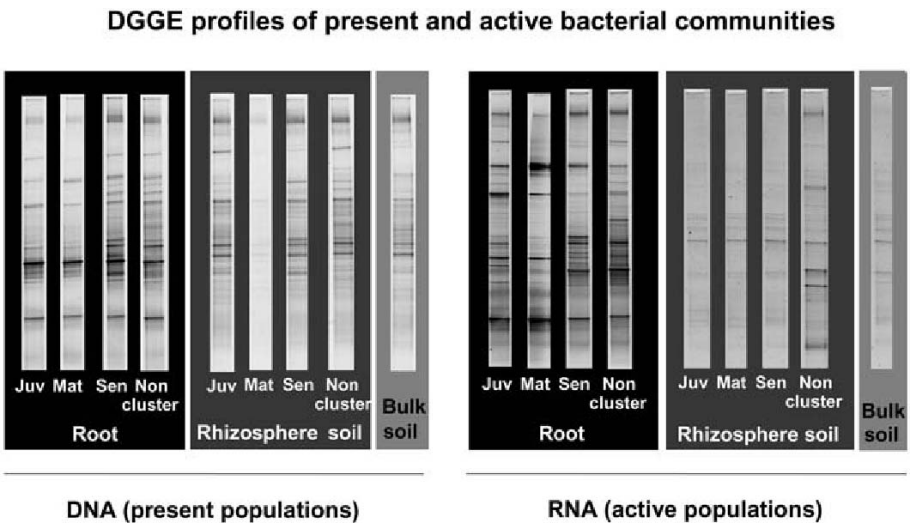

Figure 3. Example of DGGE profiles of the present (rDNA-based) and active (rRNA-based) bacterial communities at the different root stages, types and proximity.

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 micro-organisms. 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 excretion 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 excretion does not always induce acidification. On the one hand, there are cases where the excretion 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 excretion, 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., 1982a). From this point of view, it is not surprising that mature cluster

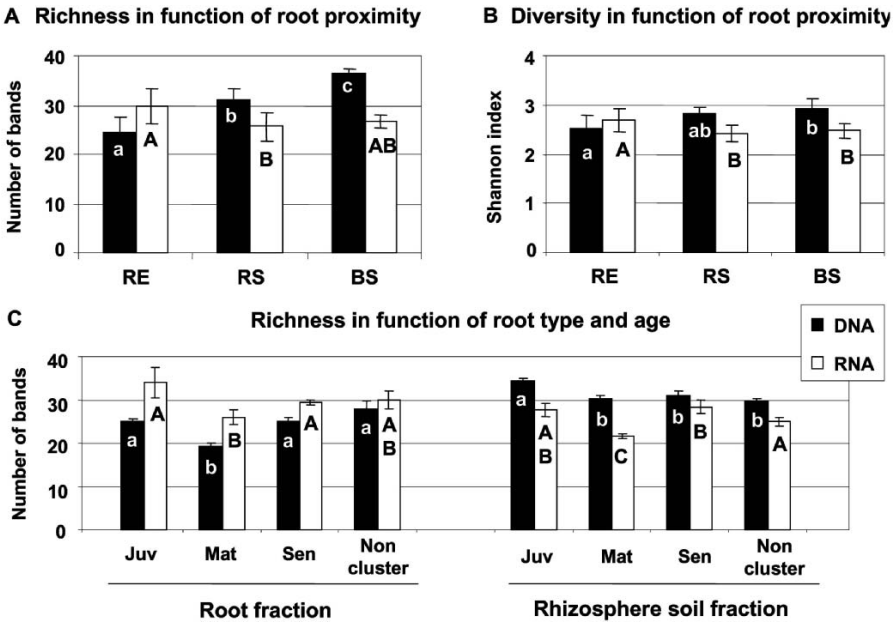

Figure 4. Richness and diversity of the bacterial communities according to root proximity, type and age. Richness was determined by counting the number of bands per DGGE profile and Shannon index was calculated as $-\sum p_i \log_2(p_i)$ where pi is the relative abundance of a given band in the profile. 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.

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 4A and 4B) . 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 5A). 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

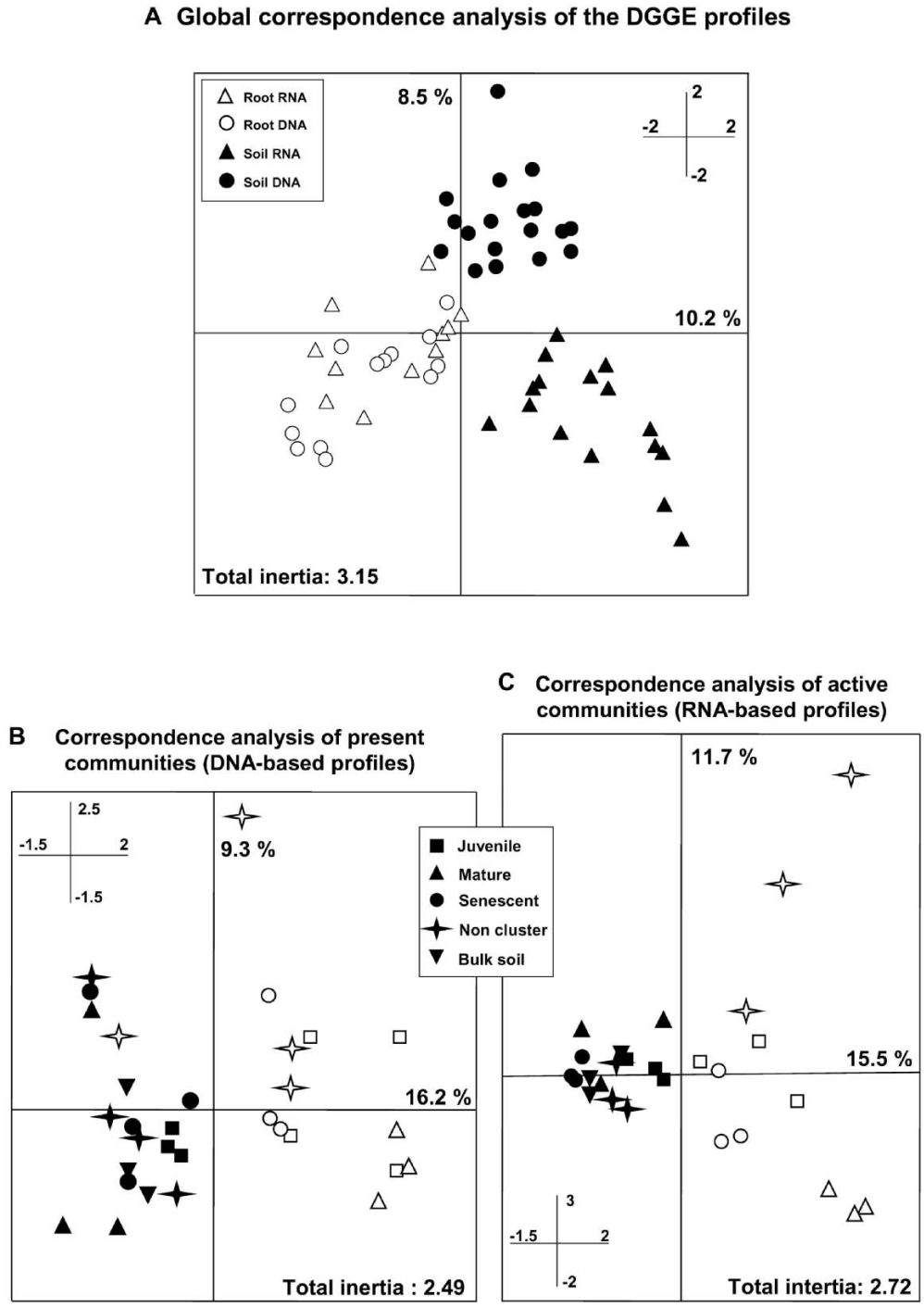

Figure 5. Correspondence analyses of bacterial community profiles for different root types, stages and proximity using 16S rDNA- and rRNA-based DGGE profiles ($n = 3$–4). Explanation percentage of the axes are calculated as eigenvalue*inertia^{-1}. A. Global analysis of rDNA- and rRNA-based samples. 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 axes 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.

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 4C). 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 excretion. 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 5C) than in present communities (Figure 5B). 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 phophorus 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, (iii) or 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 min. 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.

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