White lupin has developed a complex strategy to limit microbial degradation of secreted citrate required for phosphate acquisition

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ABSTRACT

White lupins (*Lupinus albus* L.) respond to phosphate deficiency by producing special root structures called cluster roots. These cluster roots secrete large amounts of carboxylates into the rhizosphere, mostly citrate and malate, which act as phosphate solubilizers and enable the plant to grow in soils with sparingly available phosphate. The success and efficiency of such a P-acquisition strategy strongly depends on the persistence and stability of the carboxylates in the soil, a parameter that is influenced to a large extent by biodegradation through rhizosphere bacteria and fungi. In this study, 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 of organic acid excretion and a drastic pH decrease is observed. Excretion 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 excretion. Therefore, our results suggest that white lupin has developed a complex strategy to reduce microbial degradation of the phosphate-solubilizing agents.

Key-words: *Lupinus albus*; chitinase; glucanase; isoflavonoids; phenolics; protection.

INTRODUCTION

In nature, plants often grow in soils where nutrient availability is low. As an adaptation to nutrient deficiency, 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 carboxylates such as citrate or malate. Secretion of carboxylates in response to phosphate deficiency has been reported for many plant species (Imas *et al*. 1997; Zhang, Ma & Cao 1997; Kirk, Santos & Santos 1999). In addition to this mechanism, some plant species, for example, white lupin and most members of the *Proteaceae* family, form special root structures called cluster or proteoid roots (Purnell 1960; Dinkelaker, Hengeler & Marschner 1995; Neumann & Martinoia 2002; Lamont 2003; Shane & Lambers 2005). These cluster roots strongly acidify the surrounding rhizosphere and secrete large amounts of organic acids, mainly citrate, during a short time span (3–5 d). This strategy enables the plant to efficiently extract Pi from a restricted volume of the soil. Protons solubilize P in calcareous soils. However, white lupin and also most *Proteaceae* naturally grow on acid soils. Dicarboxylates and tricarboxylates act as exchanger anions for phosphate, liberating it from Fe–P, Al–P or Ca–P complexes. The secretion of protons (which probably are exported to avoid cytosolic acidification and to maintain charge balance) and organic acids allows plants that form cluster roots to survive in soils with sparingly available phosphate. However, the efficiency of carboxylate secretion in phosphate acquisition depends to a large extent on the stability and persistence of these compounds in the soil, which is mainly influenced by the biodegradation activity of soil microorganisms. Bacteria and fungi readily take up and metabolize organic acids (Jones, Prabowo & Kochian 1996; Strom *et al*. 2001; van Hees *et al*. 2005). Thus, for plants, an efficient strategy in phosphate acquisition would require an abundant excretion of carboxylates to compensate for the loss by microbial degradation and/or a strategy to limit microbial degradation of organic acids. The latter would imply that plants implement mechanisms reducing growth and viability of micro-organisms 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 of whether or not plants

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also developed 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 frequently been used to study processes linked to cluster root function (Gardner, Parbery & Barber 1982, 1983; Johnson, Allan & Vance 1994; Neumann *et al*. 1999; Neumann *et al*. 2000; Shane *et al*. 2003; Veneklaas *et al*. 2003; Zhang, Ryan & Tyerman 2004). In white lupin, the formation of the bottlebrush-like cluster roots follows a well-defined developmental pattern (Watt & Evans 1999; Massonneau *et al*. 2001). Young, growing cluster roots release mainly malate, and only small amounts of citrate, while immature cluster roots secrete similar amounts of both. In contrast, mature cluster roots secrete far greater 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 are expected mainly at this root stage, or shortly before.

The aim of this study was to investigate if white lupin has strategies to protect secreted organic acids from microbial degradation. We show: (1) a decreased total bacteria abundance around mature cluster roots; (2) an increased isoflavonoid excretion, inducing fungal sporulation, in the juvenile and immature stage; and (3) an increased glucanase and chitinase activity at the stage preceding the excretion of citrate. Our results suggest that white lupin exhibits a complex strategy to protect secreted organic acids against microbial degradation.

MATERIALS 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). The microcosms were used in three replicates consisting of one plant planted in one microcosm. Plants were grown in inoculated sand as described previously (Weisskopf *et al*. 2005), except that the inoculation solution was prepared from a lupin field soil collected in Monte Gargano, Foggia, Italy. After 6 weeks of growth, the plants were harvested, and the different root stages were 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 (SPB) 0.1 M pH 7 , and ground with mortar and pestle. One part of the ground roots 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, with one replicate consisting of five boxes containing 12 plants each. 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 of immature and mature roots. Therefore, we were able to separately collect juvenile, immature, mature and senescent cluster roots.

Adsorption and degradation of carboxylates in the soil

Mixtures of organic acids in a concentration range detected in the rhizosphere of cluster roots (malate 7.5 mM; citrate 2.1 mM) and water controls were incubated at 20% soil moisture level with the test soils (250 mg per sample, taken from planted culture vessels to introduce an active soil microflora) using incubation times of 3 and 24 h under axenic and non-axenic conditions. Axenic conditions were obtained by chloroform fumigation of the soil samples with a 24 h pre-incubation period under a chloroform atmosphere. After 3 and 24 h, water-soluble organic acids were re-extracted with 450 μL H_2O. The soil was removed by centrifugation and organic acids in the supernatant were determined by high-performance liquid chromatography (HPLC) (Neumann *et al*. 1999). Recovery of organic acids was calculated in comparison with the water controls. Soil characteristics: Arenosol, West Africa, pH ($CaCl_2$) 4.6; P (CAL) 3 mg kg^{-1}. The influence of the buffer pH on organic acid recovery was analysed using radio-labelled citric acid. Citric acid [30 nCi (^{14}C)], Moravek Biochemicals, Brea, CA, USA, specific activity of 108 mCi $mmol^{-1}$, citrate corresponding to a final concentration of 1 mM] was incubated with 2 g of soil with a moisture of 20% for 1 h. A washing step (incubating the soil with 20 mM phosphate buffer, pH 7 for 20 min with gentle shaking) was carried out twice to eliminate the non-adsorbed citric acid. Extractions were performed with phosphate buffers at pH 7 or 3.2, as well as with 5 mM NaCl and 5 mM HCl. After 30 min of incubation, aliquots of the samples were collected and quantified by scintillation counting. The released citrate was expressed as a percentage of bound citrate. The experiments were repeated thrice, providing similar results.

Isolation of strains and growth in low- pH media

The ground roots from the three different stages of cluster roots were 10-fold serially diluted, and spread on Angle medium (Angle, McGrath & Chaney 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. A specific buffer was used for each pH value. The experiment used 0.1 M NaOH (A), 0.1 M $C_8H_5KO_4$ (B), 0.1 M HCl (C) and water (D) 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. Ten millilitres of the corresponding buffer was added to 250 mL of medium prior to autoclaving. The low-pH assay was performed in liquid cultures, using microplates (NUNC, Rochester, NY, USA). Results (growth or no growth) were recorded after 7 d of incubation at room temperature.

Total cell counts after 4′-6-diamidino-2-phenylindole (DAPI) staining

After washing off the rhizosphere soil by gently shaking in SPB, the roots from the three different stages of cluster roots were ground. One millilitre of the ground roots was added to 9 mL of 50% ethanol (v/v) for fixation. The samples were incubated for 4 min 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 2 min at 500 *g* to allow sedimentation of remaining soil and sand particles. After diluting 1:1 with 50%, ethanol (v/v), 1 mL of the supernatant was filtered on polycarbonate filters (13 mm diameter, 0.2 μm pore size) placed on nitrocellulose filter supports (13 mm diameter, 0.2 μm pore size). The samples were then stained with 70 μL DAPI (5 $\mu g\ mL^{-1}$ solution, Sigma, St. Louis, MO, USA) for 5 min in the dark. Excess DAPI was removed, and the filters were observed with a Leica Dialux microscope (Leica Microsystems Ltd, Milton Keynes, UK) at a 1000 × 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 the calculation of the total cell counts g^{-1} root fresh weight (FW).

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 4 mL of water for 1 h at room temperature with gentle shaking to allow the collection of root exudates. We have previously shown that there is no significant difference in the quality or quantity of exudates released into distilled water compared to those released into 0.5 mM $CaSO_4$, which is often used for membrane stabilization (Neumann *et al*. 1999). We used this hydroponic system to allow the separation and comparison of well-defined cluster root stages, but we were aware that the secreted compounds recovered during this experimental procedure might be different from those obtained in the field conditions, where soil micro-organisms likely influence the root secretions. In order to verify that the pattern of isoflavonoids collected was not affected by microorganisms present in the soil, we analysed the isoflavonoids secreted from lupin seedlings grown in the presence of different bacteria and fungi. The amounts of specific isoflavonoids released changed, but not the composition (data not shown). The root exudates were collected and frozen at –80 °C. The remaining roots were then incubated in 4 mL of 80% methanol (v/v) for 1 h at room temperature with gentle shaking to recover the internal cell contents. Internal isoflavanoid extracts were filtered at 0.45 μm (Schleicher & Schuell, D–37586 Dassel, Germany) and resuspended in the first HPLC solvent according to the root FW (1.5 $\mu L\ mg^{-1}$ root FW). The frozen exudates were freeze-dried and subsequently extracted with 2.5 mL of 80% methanol (v/v) in sequential steps (1 mL and subsequently thrice 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 FW (0.75 $\mu L\ mg^{-1}$ root FW). Of the samples, 50 μL (internal contents and exudates) were then injected into a reversed phase C 18 column (Nucleosil 250 × 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 min, with a flow rate of 0.4 $mL\ min^{-1}$. The absorbance was monitored at 263 nm. All analyses were performed with three to four replicates, with each replicate representing the harvest of five boxes containing 12 plants each.

Flavonoid staining with diphenyl-boric acid dimethyl amino-esther (DPBA)

The entire root system of white lupin plants grown in hydroponic culture under P-deficient conditions was placed between two agar gel layers containing 5‰ of DPBA (Sigma). After 20 min of incubation in the dark, the stained root parts were investigated under UV light (360 nm) exposure. The same staining procedure was applied to single roots.

Effects of isoflavonoids on the sporulation of *Fusarium oxysporum*

Phenolic compounds [100 μg in ethanol in 20 μL 50% ethanol (v/v)] were separated on thin-layer chromatography (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 [50% ethanol (v/v)] was used as a control. A spore suspension of the lupin pathogen *F. 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 potato–dextrose–agar (PDA) 10-fold 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 under P-deficient conditions. Four to seven replicates were used, consisting of one box containing 12 plants. After the separation of the different cluster root stages, excised root samples were incubated for 30 min at room temperature in 5 mM $CaCl_2$ (pH 6.4) with gentle shaking. The extracellular fraction was collected, 1 mg mL^{-1} bovine serum albumin (BSA) was added and samples were frozen at –80 °C prior to lyophilization. The lyophilized samples were resuspended in a 0.1 M sodium acetate buffer pH 5.

Chitinase activity

Chitinase activity was determined with dye-labelled 6-0-carboxymethyl-chitin (chitin azure, Sigma) according to Wirth & Wolf (1990) after optimization of incubation time and enzymatic extract concentration. Each reaction mixture 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 1 h of incubation at 30 °C with gentle shaking, 200 μL 0.1 M HCl was added to stop the reaction, and the samples were centrifuged for 5 min at 1800 *g*. The absorbance was read at 560 nm. For each sample, the absorbance value of the negative control (lacking chitin azure) was deducted from the final absorbance. Chitinase activity was expressed as the absorbance at 560 nm root FW^{-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 without laminarin was used to account for the colour changes between different root ages. After 1 h of incubation at 30 °C with gentle shaking, 150 μL was collected, and released glucose was determined using the Boehringer Mannheim D-Glucose determination kit (Boehringer Mannheim, Germany). Glucanase activity was expressed as mg glucose g^{-1} root FW h^{-1}.

Statistical analyses

Microscopic counts were compared statistically using Student's *t*-test ($P < 0.01$). The results of bacterial growth in low-pH media were validated using a Chi-square test (χ^2-test) ($P < 0.05$). For phenolic contents and enzyme activities, 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).

RESULTS

Carboxylates are adsorbed to the soil and degraded by micro-organisms

In order to test whether carboxylates are rapidly degraded by soil micro-organisms, we added malate and citrate to an acidic soil in concentrations reported for the rhizosphere soil of cluster roots. As shown in Table 1, most of the citrate and malate were adsorbed to the soil matrix. Adsorption was differentially expressed for malate and citrate, and accounted for 55–70% of the recovery of carboxylates. After 3 h, the same recovery was obtained under axenic and non-axenic conditions, indicating that at this time scale, microbial degradation was negligible. Moreover, carboxylate concentrations in the soil solution rapidly reached equilibrium concentrations, because recovery after 3 and 24 h axenic incubation revealed similar values. This is in line with observations of Jones *et al*. (2003).

The impact of biodegradation on carboxylate recovery increased with incubation times longer than 3 h, leading to complete degradation of the extractable organic acids within 24 h, even at the high concentration levels accumulating in the rhizosphere of cluster roots. Because it must be assumed that an equilibrium between bound and free carboxylates is established, microbial degradation will result in partial release of bound carboxylates, and consequently, biodegradation is probably underestimated. Because white lupin acidifies the rhizosphere, we tested if the use of an acidic buffer (pH 3.2) would release more organic acids. This was indeed the case (about 6% at pH 7 and 12% at pH 3.2), increasing the organic acid fraction

Table 1. Recovery of water-extractable citrate and malate after incubation in axenic versus non-axenic conditions

	Recovered by water extraction (%)			
Carboxylates	3 h axenic	3 h non-axenic	24 h axenic	24 h non-axenic
Malate (7.5 mM)	46.7 ± 4.0	44.5 ± 4.2	34.8 ± 1.7	0
Citrate (2.1 mM)	32.5 ± 3.5	29.5 ± 1.9	12.8 ± 11.1	0

Recovery of water-extractable organic acids applied in concentrations reported for the rhizosphere soil solution of cluster roots (Neumann & Römheld 2000) at a soil moisture level of 20% and different incubation times under axenic (chloroform atmosphere) and non-axenic conditions. Means and SD of three independent replicates.

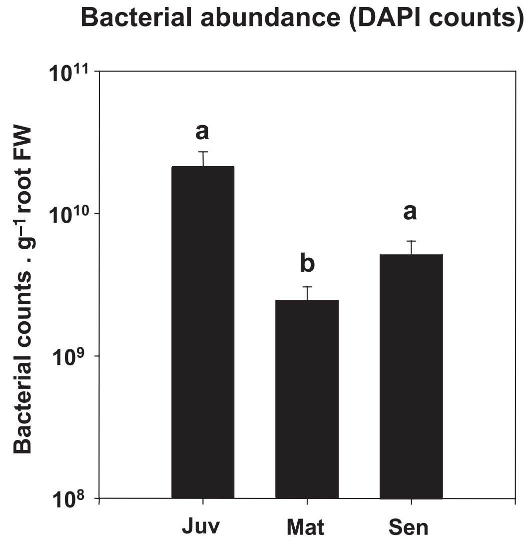

Figure 1. Numbers of bacteria g^{-1} fresh weight (FW) as a function of cluster -root stage. Bacterial abundance was determined by microscopic counts after DAPI staining.
Juv, juvenile; Mat, mature; Sen, senescent. Values with different letters (a, b) are significantly different (Student's *t*-test,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

available for microbial degradation. These findings emphasize the importance of mechanisms that counteract the biodegradation of carboxylates for an efficient P-mobilization, which usually requires mM concentrations of carboxylates in the extraction solution (Neumann & Römheld 2000).

Acidification at the mature cluster root stage decreases bacterial abundance

In a recent paper (Weisskopf *et al.* 2005), we showed that the number of bacteria that could be cultivated was significantly reduced at the mature stage of cluster roots. In order to verify if this reduced abundance deduced from plate counts was not a result of a decrease in the capacity of the different bacteria to be cultivated, we performed microscopic counts after DAPI staining of bacteria collected at different root stages. In agreement with what was observed for bacteria that could be cultivated, total bacterial abundance decreased significantly at the mature stage of cluster roots, which corresponds with the stage of fastest citrate excretion (Fig. 1).

A possible explanation for this decrease could be the low pH (4 or below) associated with the mature stage of cluster roots, because of a concomitant release of protons and citrate excretion. To test this hypothesis, we assessed the ability of isolated strains to grow at pH conditions ranging from pH 3 to 7. The ability of about 100 strains isolated from three stages of white lupin cluster roots to grow on an acidic medium (pH 4) is shown in Fig. 2. The proportion of strains able to grow at pH 4 was significantly higher for mature stage cluster roots than for the juvenile stage ones, suggesting that because of 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 among the different stages were observed at the other pH values (data not shown).

Prior to citrate excretion, isoflavonoids are secreted in large amounts into the rhizosphere

Because fungi are more tolerant to acidic pH compared to bacteria, we investigated other possible defence mechanisms involved in fungal growth inhibition. It has been reported previously that cluster roots secrete phenolics (Neumann *et al.* 1999), which might act as antifungal compounds. The white lupin cluster roots secreted large amounts of phenolic compounds, and most of these phenolics were isoflavonoids. The internal concentration of isoflavonoids significantly increased from the juvenile to the immature stage (Fig. 3a). The excretion pattern followed the same trend with the largest amounts secreted at the juvenile and immature stages, the root stages preceding the citrate burst (Fig. 3b). Genistein was the isoflavonoid secreted in largest amounts (up to 0.8 mg g^{-1} root FW h^{-1} from immature cluster roots, data not shown), and most other compounds were conjugates of genistein (Weisskopf *et al.*, unpublished results).

This pattern of isoflavonoids in the different stages of white lupin cluster roots was confirmed by an *in situ* staining of the entire root system with DPBA, a flavonoid-specific dye (Fig. 3c). Young and immature cluster roots

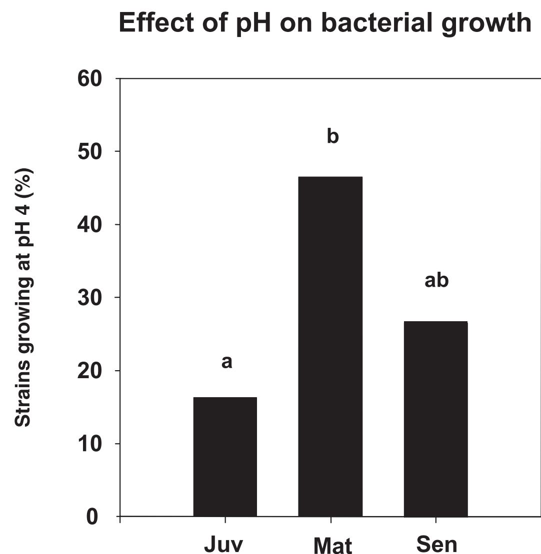

Figure 2. Frequencies (in percentage of total isolated strains) of strains growing in a low-pH (4) medium. About 100 strains were randomly picked, at least 30 per root stage.
Values with different letter (a, b) are significantly different [(Chi-square test (χ^2-test), $P < 0.05$, $n \geq 30$]).
Juv, juvenile; Mat, mature; Sen, senescent.

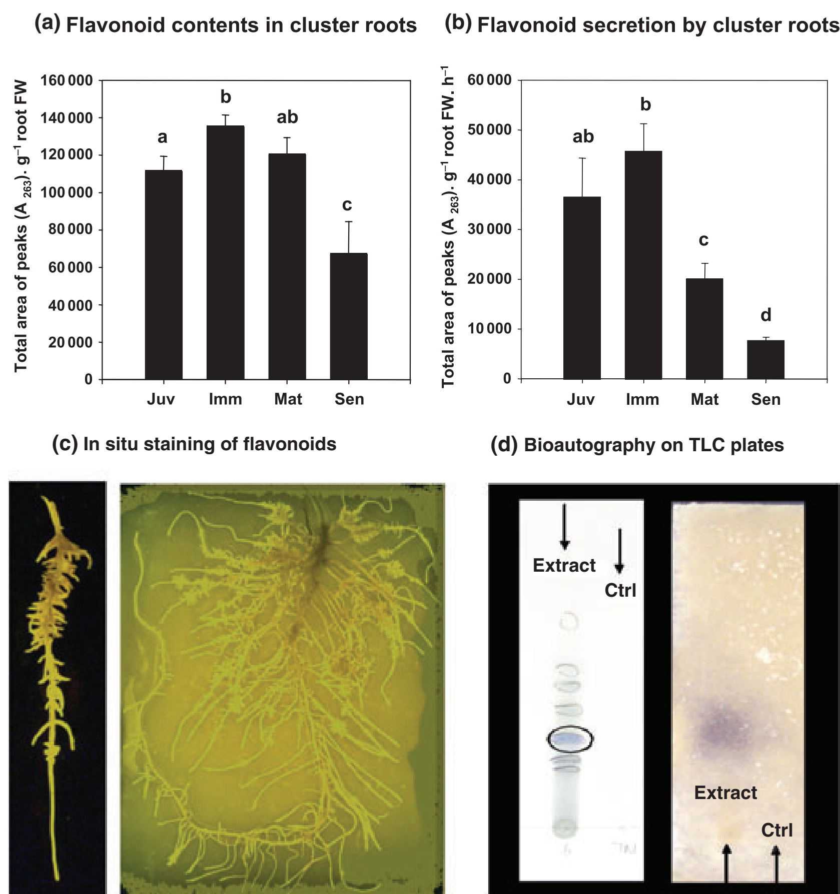

Figure 3. Flavonoids in white lupin cluster roots. Flavonoids produced in (a) and secreted from (b) different stages of cluster roots. Juv, juvenile; imm, immature; mat, mature; sen, senescent. Total amounts were calculated as the total area of all peaks measured by UV detection (263 nm) after high-performance liquid chromatography (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, $P < 0.05$, $n = 3$). (c) *In situ* staining of flavonoids with DPBA (diphenyl-boric acid dimethyl amino-esther (DPBA)). White lupin roots (single cluster roots: left picture; 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 min of incubation, the roots were exposed to UV light (360 nm). (d) Effects of flavonoids secreted by white lupin cluster roots on the sporulation of *Fusarium oxysporum*. Left panel: 50 μg of phenolic extracts were separated on a thin-layer chromatography (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 genistein. A 50 μg part of phenolic extracts was used for this assay. Ctrl: negative control (only solvent). Picture was taken 5 d after inoculation. See Material and Methods section for details.

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. Because 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 rhizosphere, as well as some collection strains, which represented potential pathogens of white lupin. Among the tested fungi, some showed an increased sporulation when exposed to the phenolics secreted by white lupin. Because active growth and nutrition occur in fungi only during the vegetative stage (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 *F. oxysporum* showed the strongest reaction, and was chosen as a biotest strain for further investigations. In a bioassay based on TLC plate bioautography (Fig. 3d), we observed that stimulation of sporulation occurred approximately at the height (Rf) as the isoflavonoid with the highest UV absorption, identified as malonyl genistein. Unfortunately, we were not able to observe this stimulation of the sporulation when fractions were fur-

ther purified, suggesting that more than one compound migrating at a similar Rf value as malonyl genistein were involved in this phenomenon, and that they might act in a synergistic way. No inhibitory effect of these isoflavonoids on bacterial growth was observed.

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

A widespread defence mechanism of plants against a broad spectrum of fungi is the secretion of cell wall-degrading enzymes into the rhizosphere. We have shown in a previous study that with a complementary DNA amplified fragment length polymorphism (cDNA–AFLP) assay, several genes involved in carbohydrate metabolism were differentially expressed during cluster root formation under 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. The sequences of these genes were clearly plant-derived, and did not correspond to microbial chitinases. To verify that the higher level of gene induction corresponded to an enhanced activity, we measured extracellular glucanase and chitinase activities at the different cluster root stages. Chitinase and glucanase activities were both significantly ($P < 0.05$) higher in the immature stage of cluster roots (Fig. 4) compared to all other stages.

The cultivation technique used keeps microbial growth very low. This is reflected by the fact that secreted malate and citrate does not significantly decrease when keeping the samples for 60 min at room temperature, excluding the fact that microbial chitinases influenced the measured enzymatic activities. Furthermore, a semiquantitative reverse transcriptase-PCR for glucanase transcripts revealed a similar pattern as the enzymatic activities (not shown). These results show that the release of chitinase and glucanase also followed a developmental pattern similar to organic acid and isoflavonoid release.

DISCUSSION

In natural environments, phosphate is often a factor that limits plant growth. To deal with this situation, most plants form mycorrhizal associations, which enable them to survive in soils with sparingly available phosphate. An alternative, very efficient strategy to mobilize phosphate from soils and is used mainly by non-mycorrhized plants, is the formation of proteoid roots. In contrast to the phosphate-acquisition strategy of mycorrhizae, cluster root-bearing plants secrete large amounts of carboxylates in a restricted volume of soil at a short time span. The carboxylates serve as exchange anions to solubilize sparingly available phosphate in proximity of the rootlets. The problem of this strategy could be that micro-organisms might efficiently take up and metabolize carboxylates. There was no significant difference between juvenile, mature and senescent cluster root stages when comparing the percentage of isolated strains that are 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, in general, are more sensitive to acidic environments than fungi, and a transient decrease of the rhizosphere pH should limit their activity. This transient pH decrease is precisely what happens at the mature stage of

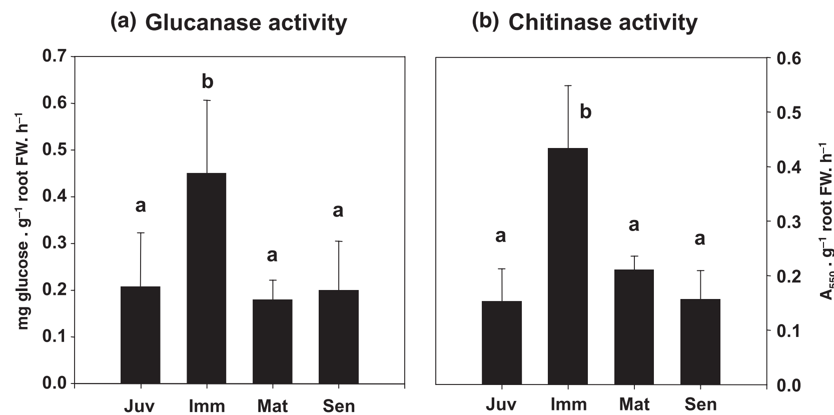

Figure 4. Activities of cell -wall- degrading enzymes at the different cluster -root stages. Juv, juvenile; imm, immature; mat, mature; sen, senescent. (a) Glucanase activity was measured spectrophotometrically by determining the amount of glucose liberated from laminarin during a one-hour incubation period. (b) Chitinase activity was measured spectrophotometrically using the dye-labelled chitin -azure for 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, $P < 0.05$, $n = 4–7$).

cluster roots, which can acidify the rhizosphere to a pH of 4 and below. We showed that the bacterial population levels transiently decreased 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 with the excretion of organic anions may have several roles: (1) participating in the maintenance of a charge balance through compensation of the secreted negatively charged citrate (Neumann *et al*. 1999; Zhu *et al*. 2005); (2) solubilizing P in calcareous soils; (3) providing an optimal pH for the secreted hydrolases, such as chitinase, glucanase or acid phosphatase, and as suggested by this study; (4) 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 efficiency of white lupin cluster roots. Because fungi usually are better able to cope with acidic conditions, other mechanisms than rhizosphere acidification are required to inhibit fungal growth. Some phenolics, like flavonoids or isoflavonoids, display antifungal activities (Tahara *et al*. 1994; Dakora & Phillips 1996; Weidenborner & Jha 1997). White lupin produces a large amount of different isoflavonoids (Stobiecki *et al*. 1999; Katagiri, Ibrahim & Tahara 2000) and some of them act as antifungal compounds (Wojtaszek & Stobiecki 1997; Bednarek *et al*. 2003). We found that isoflavonoids are secreted from cluster roots of white lupin. We also showed that white lupin isoflavonoids induced sporulation in several fungal strains. Sporulation is often a stress response in fungi, and because spores, in contrast to mycelium, do not cause degradation of citrate and malate, this stimulation of sporulation can be viewed as a mechanism to protect 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 large amount of organic acids in order to sporulate. However, the fact that the isoflavonoid burst occurs prior to the citrate excretion suggests that sporulation takes place before organic acids are secreted and could be consumed. When spores germinate, the newly developed mycelium could then again degrade organic acids. However, it is unlikely that this occurs at the short time span of cluster root development and organic acid secretion (2–4 d). Interestingly, among the fungi tested, *F. oxysporum* was the most susceptible to white lupin isoflavonoids. *Fusarium* species have been previously reported to be inhibited by flavonoids (Silva, Weidenborner & Cavaleiro 1998), and to elicit isoflavonoid accumulation in soybean roots (Lozovaya *et al*. 2004). Furthermore, *Fusarium* species are well-known pathogens of lupins (Bateman 1997; Satyaprasad, Bateman & Ward 2000; Shield *et al*. 2000).

In addition to this increased isoflavonoid excretion, white lupin apparently 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 play a role in plant defence against pathogenic fungi, in white lupin as well as in other plant species (Vierheilig *et al*. 1994; Burzynski, Pislewska & Wojtaszek 2000; Tonon *et al*. 2002). However, because these enzymes are involved in fungal cell wall degradation, their inhibitory effect will not only be limited specifically to pathogenic fungi, but will also affect other rhizosphere fungi, which may use citrate and malate as carbon sources. In white lupin cluster roots, we showed that both chitinase and glucanase had a higher activity at the immature stage, the stage preceding the rapid citrate excretion.

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

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