

Isoflavonoid exudation from white lupin roots is influenced by phosphate supply, root type and cluster-root stage

Laure Weisskopf¹, Nicola Tomasi¹, Diana Santelia¹, Enrico Martinoia¹, Nicolas Bernard Langlade¹, Raffaele Tabacchi² and Eliane Abou-Mansour²

¹Laboratory of Molecular Plant Physiology, Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, 8008 Zürich, Switzerland;

²Laboratory of Analytical Organic Chemistry, Institute of Chemistry, University of Neuchâtel, Avenue Bellevaux 51, 2007 Neuchâtel, Switzerland

Summary

- The internal concentration of isoflavonoids in white lupin (*Lupinus albus*) cluster roots and the exudation of isoflavonoids by these roots were investigated with respect to the effects of phosphorus (P) supply, root type and cluster-root developmental stage.
- To identify and quantify the major isoflavonoids exuded by white lupin roots, we used high-pressure liquid chromatography (HPLC) coupled to electrospray ionization (ESI) in mass spectrometry (MS).
- The major exuded isoflavonoids were identified as genistein and hydroxygenistein and their corresponding mono- and diglucoside conjugates. Exudation of isoflavonoids during the incubation period used was higher in P-deficient than in P-sufficient plants and higher in cluster roots than in noncluster roots. The peak of exudation occurred in juvenile and immature cluster roots, while exudation decreased in mature cluster roots.
- Cluster-root exudation activity was characterized by a burst of isoflavonoids at the stage preceding the peak of organic acid exudation. The potential involvement of ATP-citrate lyase in controlling citrate and isoflavonoid exudation is discussed, as well as the possible impact of phenolics in repelling rhizosphere microbial citrate consumers.

Key words: genistein, isoflavonoids, *Lupinus albus* (white lupin), phosphate deficiency, proteoid roots.

Introduction

In nature, plants often grow in soils with low available phosphate. To cope with this problem, they have developed several resistance mechanisms (Schachtman *et al.*, 1998; Raghothama, 1999). Two main strategies are the association with mycorrhizal fungi and the formation of particular root structures called 'cluster roots' or 'proteoid roots' (Purnell, 1960). In most cases, plants form either cluster roots or mycorrhizas, but more and more exceptions are being found to this rule of mutual exclusion of the two strategies (Shane & Lambers, 2005a and references

therein). Cluster-root formation is not as common in plants as mycorrhizal symbioses, but it is a very efficient strategy to cope with phosphate deficiency and does not depend on the availability of the fungal partner. Cluster roots release large amounts of organic acids into the rhizosphere (Neumann & Martinoia, 2002; Lamont, 2003; Shane & Lambers, 2005a; Le Bayon *et al.*, 2006) and these organic acids solubilize phosphate by chelation and ligand exchange (Dinkelaker *et al.*, 1989, 1997; Gerke *et al.*, 1994, 2000; Jones & Darrah, 1994; Ryan *et al.*, 2001; Dakora & Phillips, 2002). Cluster-root physiology and ecology have been intensively studied over the last decade

(Skene, 1998; Watt & Evans, 1999; Neumann & Martinoia, 2002; Lamont, 2003; Shane & Lambers, 2005a,b) and particular interest has been devoted to the exudation of organic acids. However, not much attention has been paid so far to other exuded molecules such as phenolics, despite their potential role in plant nutrition (Dinkelaker *et al.*, 1995; Marschner, 1995) and plant–microbe interactions (Dakora & Phillips, 1996; Paiva, 2000).

Accumulation of phenolics is a well-known symptom of nutrient stress, and different classes of phenolic compounds are produced depending on the nature of the stress: phosphate deficiency is known to induce anthocyanin accumulation, while the production of phenolics may be up-regulated under iron starvation, and flavonoids, as well as isoflavonoids, have been linked to nitrogen stress (Dixon & Paiva, 1995). Previous reports have studied the impact of phenolics on metal resistance, with resistance to aluminium being investigated in maize (*Zea mays*) (Kidd *et al.*, 2001) and resistance to copper in alfalfa (*Medicago sativa*) (Parry & Edwards, 1994) and white lupin (*Lupinus albus*) (Jung *et al.*, 2003). Jung and coworkers showed that isoflavonoids are able to bind copper ions and speculated 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 rhizosphere acidification. For many heavy metals, solubility increases when pH decreases and phenolics might help to reduce this side effect of phosphate solubilization.

In addition to their role in iron nutrition (Römheld & Marschner, 1983; Zhang *et al.*, 1991; Moran *et al.*, 1997) or heavy metal resistance (Schutzendubel *et al.*, 2001; Jung *et al.*, 2003), phenolics, especially flavonoids, also play a major role in plant–microbe interactions (Paiva, 2000). In legumes, isoflavonoids are an 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 such as nitrogen-fixing bacteria (Dakora *et al.*, 1993) and mycorrhizal fungi (Hirsch & Kapulnik, 1998). On the other hand, many isoflavonoids are involved in the defence 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 & Phillips (1996).

Previous studies on isoflavonoids have been conducted in the cluster-rooted species white lupin (*Lupinus albus* L., Fabaceae). However, these studies either focused on the precise profiling and structural elucidation of the various isoflavonoids in white lupins grown in phosphorus (P)-sufficient conditions, and thus without cluster roots (Wojtaszek *et al.*, 1993; Wojtaszek & Stobiecki, 1997; Katagiri *et al.*, 2000, 2001; Sakasai *et al.*, 2000; Bednarek *et al.*, 2001, 2003; Pislewska *et al.*, 2002; Jung *et al.*, 2003), or took phenolics into account only as a general class of compounds in cases where P-deficient white lupins forming cluster roots were studied (Dinkelaker *et al.*, 1995; Neumann *et al.*, 2000). To date, no study has investigated the

isoflavonoids produced and exuded by the cluster roots of white lupin plants.

In white lupin, cluster roots can be separated into four different growth stages (Massonneau *et al.*, 2001): the juvenile, the immature, the mature and the senescent stages. These stages differ in the quantity and quality of organic acid exudation: at the juvenile stage, cluster roots are still growing and secrete mainly malate. After 2–3 d, the cluster root reaches the immature stage, where the full size of the root is achieved but no or little exudation of organic acids occurs. High rates of exudation of organic acids, mainly citrate, occur at the mature stage. At this point, a concomitant release of protons causes rhizosphere acidification. However, acidification and carboxylate exudation are two separate processes. Carboxylates are exuded in the unprotonated form through recently identified channels (Kollmeier *et al.*, 2001; Sasaki *et al.*, 2004; Zhang *et al.*, 2004), whereas acidification is a result of the activation of the plasma membrane proton pump (Yan *et al.*, 2002). In addition to protons, sodium and potassium ions have been shown to serve as counterions for citrate release and may be quantitatively more important than protons for the maintenance of charge balance (Zhu *et al.*, 2005). At the senescent stage, almost no carboxylates are exuded.

In the present work, we aimed to extend our knowledge of the physiology of cluster-root exudation beyond the carboxylates to secondary compounds, and we chose to focus our attention on isoflavonoids because of their abundance in legumes and their potential role both in nutrition and in plant–microbe interactions. We took advantage of the development of high-pressure liquid chromatography (HPLC) coupled to electrospray ionization (ESI) in 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) Does phosphate status induce changes in the quantity and quality of the isoflavonoids produced and exuded? (ii) Is the isoflavonoid composition different in cluster and noncluster roots? (iii) Is the exudation pattern for isoflavonoids similar to that for organic acids in white lupin cluster roots?

Materials and Methods

Chemicals

The 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 (Buchs, Switzerland).

Plant material and harvest of different root parts

White lupin plants (*Lupinus albus* L. cv. Amiga; Südwestdeutsche Saatzeit, Rastatt, Germany) were grown either in the presence (+P) or the absence (–P) of a 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For the separation between cluster and noncluster roots (without differentiation of the cluster-root stages), cluster roots of all developmental stages were harvested and pooled, while noncluster 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 between 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).

Extraction of phenolic compounds and HPLC analysis

Excised root parts were washed in distilled water and subsequently incubated in 4 ml of water for 1 h at room temperature under gentle shaking to allow the exudation of root exudates (modified from Neumann *et al.*, 2000). The root exudates were collected and frozen at -80°C . After freeze-drying, 2.5 ml of 80% methanol was added in four steps (1 ml initially, followed by three additions of 0.5 ml). Each step was followed by vigorous shaking and filtration at 0.45 μm (Schleicher & Schuell, Dassel, Germany). The remaining roots were then incubated in 4 ml of 80% methanol [volume/volume (v/v)] for 1 h at room temperature under gentle shaking to recover the internal cell concentrations. Internal concentration extracts were filtered at 0.45 μm (Schleicher & Schuell). After solvent evaporation, extracts were resuspended in the first HPLC solvent (A) in proportion to the root fresh weight (FW) (0.75 $\mu\text{l mg}^{-1}$ root FW for the exudation samples and 1.5 $\mu\text{l mg}^{-1}$ root FW for the internal samples). Volumes of 50 μl were loaded on a Machery-Nagel (Düren, Germany) Nucleosil 100-5 C18 column (254 \times 4 mm) protected by a precolumn of the same material. Gradient elution was performed by varying the proportion of solvent A [acetonitrile (MeCN):H₂O:acetic acid (AcAc), 5 : 93 : 2, v/v/v] and solvent B (MeCN:H₂O: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 and 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, 264 and 320 nm. For quantification of the exuded amounts of genistein, genistein 7-*O*-diglucoside, genistein 6''-*O*-malonyl-diglucoside and genistein 6''-*O*-malonyl-*O*-glucoside, calibration curves were elaborated with isolated compounds from cluster-root exudates. Single compounds were obtained by separately collecting peaks after HPLC analysis. The peak purity was assessed by HPLC after collection of individual compounds and calibration curves were obtained based on the peak area (absorption at 263 nm). All analyses were performed with three or four replicates, each replicate representing the harvest of about five boxes containing 12 plants each.

Structural elucidation: LC-ESI-MS analysis

In leguminous plants, a small part of isoflavonoid glucosides is present as acylated conjugates (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 (Stobiecki *et al.*, 1999; Bednarek *et al.*, 2001; March *et al.*, 2004; Kachlicki *et al.*, 2005). On the basis of mass to charge ratio (m/z) values of protonated $[\text{M}+\text{H}]^+$ and fragment ions, it is possible to identify an acyl substituent linked to a sugar moiety in an isoflavonoid glycoside.

HPLC analysis was performed in an acetonitrile gradient in water using acetic acid as a 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 $[\text{M}+\text{Na}]^+$ ions in the MS, as previously observed by Stobiecki *et al.* (1999). A Hewlett Packard 1100 HPLC system (Hewlett Packard, Palo Alto, CA, USA) equipped with a binary pump was used with a photodiode array spectrophotometric detector and coupled to an Agilent 1100 series LC/MSD Trap instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with an electrospray ionization source, all controlled by the Agilent CHEMSTATION software. Separation was performed as mentioned above. For positive and negative ionization mode in ESI-MS analysis, the pressure of the nebulizer (nitrogen) was set at 50 psi (corresponding to 3.44 bar) dry gas flow at 7 ml min^{-1} and the temperature of the drying gas (N₂) at 300°C. The capillary voltage was set at 3660 V in positive and 2900 V 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 (at a concentration of 1 $\mu\text{g ml}^{-1}$ in MeCN:H₂O:AcAc; 1 : 1 : 0.02). Hydroxygenistein and genistein-7-*O*-glucoside were isolated from white lupin leaf extracts and structural identification was based on nuclear magnetic resonance and MS data according to the literature (Ingham, 1976; Ingham *et al.*, 1983; Tahara *et al.*, 1984; Murthy *et al.*, 1986).

The relative intensities of the molecular species and fragment ions were dependent on the ionization mode. Ion chromatograms resulting from LC-ESI-MS analyses of the root exudates extract are shown in Fig. 1, together with LC-UV traces obtained in parallel. In the positive ion mode analysis (Supplementary Fig. S1a), the main peak $[\text{M}+\text{H}]^+$ was observed and no fragmentation occurred. By contrast, in the negative ion mass spectra (Supplementary Fig. S1b), Y_n⁻ type fragment ions were mainly observed corresponding to the genistein moiety Y₀⁻ m/z 269 or the hydroxygenistein moiety Y₀⁻ m/z 285. The elimination of 44 atomic mass units (u) was connected with the elimination of an acetyl group leading to the fragment ion m/z 473, and the elimination of a COCH₂COO fragment (86 u) derived from malonate, giving rise to an ion at m/z 431 (genistin) and 447 (hydroxygenistin), while cleavage of the

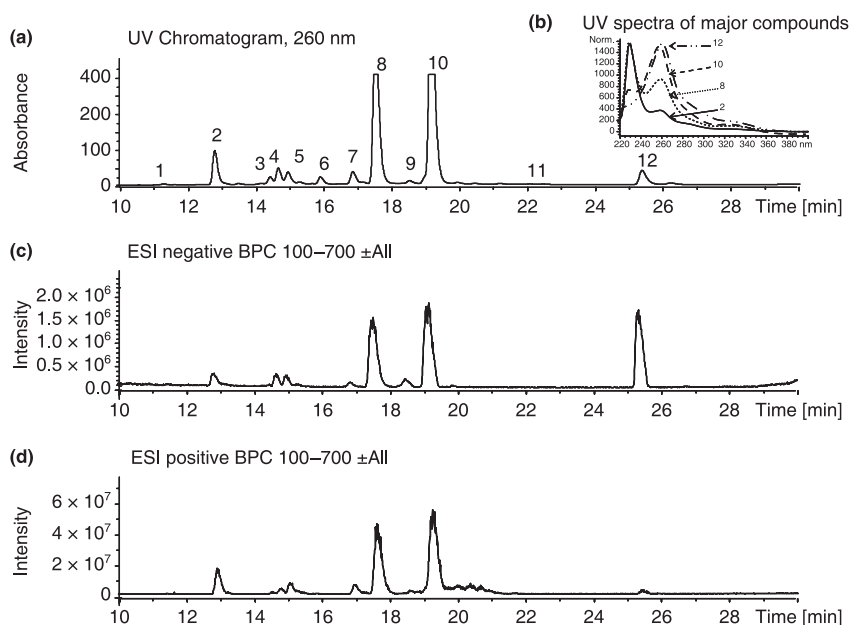


Fig. 1 Liquid chromatography (LC) and ultraviolet (UV) chemical profiles of white lupin (*Lupinus albus*) root extracts. (a) Liquid chromatography–ultraviolet (LC-UV) chromatogram; (b) UV spectra of major compounds; (c) liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) $[M-H]^-$ ion and (d) LC-ESI-MS $[M+H]^+$ ion base peak chromatograms (BPCs) of white lupin root exudate extract.

glucosidic bond by loss of 162 u [(glucose (Glc)] gave rise to the aglycone moiety ion at m/z 269 (genistein) and 285 (hydroxygenistein). The abundance of the fragments $[M-H-Mal]^-$ and $[M-H-Mal-Glc]^-$ (where Mal is malonyl) was very important compared with $[M-H]^-$, giving rise to an ion corresponding to the aglycone.

It was not possible to establish unambiguously the position of the sugar substitutions on genistein diglycoside, and two possibilities are suggested: the compound could be genistein 7-*O*-diglycoside or a structure deduced from literature data, which indicate 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 malonyl group is probably in the 6'-position of the sugar part, and this hypothesis is supported by the fact that to date only 6'-malonates of the isoflavonoid glucosides have been reported from white lupin. ESI mass spectra and fragmentation diagnostics are presented in supplementary material (Supplementary Figs S1 and S2).

Hydrolysis

Two milligrams of methanolic extract from the roots of white lupin was 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, evaporated and redissolved in 200 μ l of solvent A. A volume of 10 μ l was loaded on the HPLC column for analysis.

Statistical analyses

Analyses of variance were performed with S-PLUS 6 Statistical Software (Insightful Corporation, Seattle, WA, USA) with

a confidence interval of 95%. We used one-way analysis of variance (ANOVA) to test the general influence of P status or cluster-root stage for statistical relevance and Student's *t*-test for pairwise comparisons.

Results

Profiling of isoflavonoids in roots and root exudates

The enzymatic hydrolysis of a root exudate extract with β -glucosidase yielded only the free aglycones genistein and hydroxygenistein, establishing the β configuration of the glucosidic linkages between aglycones and sugar moieties and the absence of prenylated isoflavonoids.

The isoflavonoids were detected on the basis of their UV absorption at 263 and 350 nm (Fig. 1). Twelve peaks of isoflavonoids (Table 1), identified previously in white or yellow lupin (*Lupinus luteus*) (Shibuya *et al.*, 1991; Franski *et al.*, 1999), were tentatively recognized 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 sugars and aglycones. In addition to the free aglycones genistein (peak 12) and hydroxygenistein (peak 11), the root exudate extracts contained mono (peaks 6, 8, 9) and diglycosyl (peaks 1, 2) conjugates, as well as mono (peaks 7, 10) and diglycosyl malonyl (peaks 3, 4, 5) conjugates. Genistein conjugates were more abundant than hydroxygenistein conjugates.

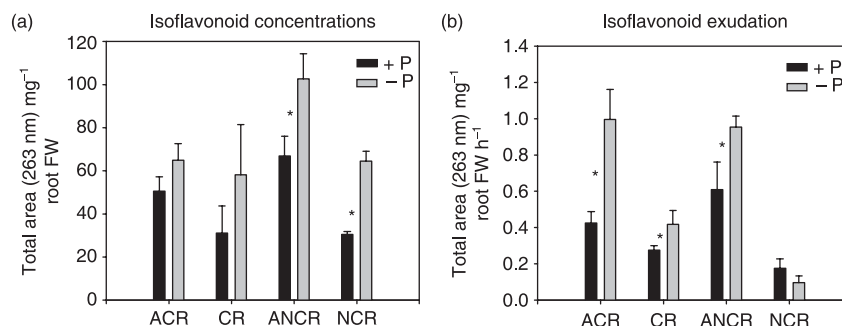
Isoflavonoid concentrations and exudation are influenced by P supply

To assess the influence of phosphate supply on the concentrations and exudation of isoflavonoids, phenolics were extracted

Table 1 Isoflavonoids and their glucosides identified in white lupin (*Lupinus albus*) root exudates

Peak number	Compound	[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

Fig. 2 Effect of phosphorus (P) supply on isoflavonoid concentrations (a) and exudation (b). White lupins (*Lupinus albus*) were grown under P-sufficient (black bars) or P-deficient (grey bars) conditions. Roots were separated into the apex of cluster roots (ACR), cluster roots (without the apex) (CR), the apex of noncluster roots (ANCR) and noncluster roots (without the apex) (NCR). Bars represent means of three replicates. *, significant differences (Student's *t*-test, $P < 0.05$). FW, fresh weight.



from white lupin plants grown either in P-deficient or P-sufficient conditions. As previously observed (Shen *et al.*, 2003), P-sufficient lupin plants also produced cluster roots, but in a much lesser abundance than P-deficient plants. Roots were separated into four fractions: (i) the apex of cluster roots, (ii) cluster roots (without the apex), (iii) the apex of noncluster roots and (iv) noncluster roots (without the apex). Isoflavonoid concentrations and exudation were analysed in the four root types. The total amounts of isoflavonoids were calculated as the sum of the areas of the major peaks in the HPLC profiles detected by UV. When grown under P deficiency, plants produced and exuded significantly more isoflavonoids (ANOVA, $P < 0.05$) than when they were supplied with phosphate (Fig. 2). For isoflavonoid concentrations (Fig. 2a), this effect of P nutrition was especially marked for noncluster roots and their apices (Student's *t*-test, $P < 0.05$), while in cluster roots and their apices no significant difference was observed between plants grown with and without phosphate. As observed for the concentrations, the exudation of isoflavonoids within the same incubation period was generally higher in plants grown in the absence of phosphate (Fig. 2b), except for noncluster roots, where no significant difference was observed.

Phosphate status did not affect all isoflavonoids to the same extent: for example, in cluster roots, a significant increase in genistein (peak 12) concentrations was observed in P-deficient plants (Student's *t*-test, $P < 0.05$). These plants contained 2.3 mg genistein g⁻¹ root FW, while only 0.9 mg g⁻¹ root FW was

detected for plants grown in P-sufficient conditions. In contrast, no effect of P treatment was observed for genistein 6'-O-malonyl-O-glucoside (peak 10), which was present at a concentration of approximately 0.2 mg g⁻¹ in cluster roots of both P-deficient and P-sufficient plants. Overall, the ratio of internal concentrations vs exudation was not altered by the P treatment.

Isoflavonoid concentrations and exudation are influenced by root type

In order to investigate the influence of root type on isoflavonoids in white lupin, we compared the isoflavonoid concentrations and exudation of cluster and noncluster roots (Fig. 3). For the internal concentrations of isoflavonoids (Fig. 3a,b), higher levels of genistein (peak 12) were found in cluster roots ($P < 0.05$) than in noncluster roots, but no significant difference was observed for the other compounds. In contrast, the exudation of isoflavonoids was generally higher for cluster roots than for noncluster roots (Fig. 3c,d).

Isoflavonoid concentrations and exudation are influenced by cluster-root stage

To assess whether the developmental stage of cluster roots plays a role in isoflavonoid storage and exudation, cluster roots were separated into juvenile, immature, mature and senescent cluster roots. For all root stages, internal isoflavonoid concentrations

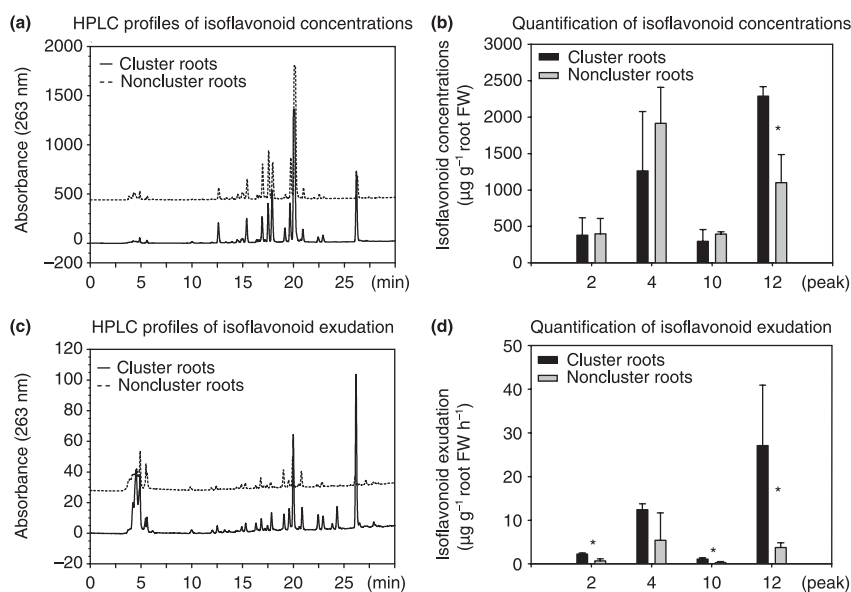


Fig. 3 Effect of root type on isoflavonoid concentrations (a, b) and exudation (c, d). White lupins (*Lupinus albus*) were grown under phosphorus (P)-deficient conditions. Roots were separated into cluster roots and noncluster roots. (a, c) High-performance liquid chromatography (HPLC) profiles of cluster roots (solid line) and noncluster roots (dashed line), for concentrations (a) and exudation (c). (b, d) Quantification of four major isoflavonoids present in and exuded from cluster roots (black bars) and noncluster roots (grey bars). peak 2, genistein 7-*O*-diglucoside; peak 4, genistein 6'-*O*-malonyl-diglucoside; peak 10, genistein 6'-*O*-malonyl-*O*-glucoside; peak 12, genistein. Bars represent means of three replicates; *, significant differences (Student's *t*-test, $P < 0.05$).

and exudation were determined. The HPLC profiles shown in Fig. 4 are representative examples of the four replicates we analysed for each stage. Only small changes were observed in the concentrations of isoflavonoids (Fig. 4a), but greater differences were observed in isoflavonoid exudation (Fig. 4b) along the growing cluster roots. In contrast with the pattern of organic acid exudation, which is highest in mature cluster roots, most of the isoflavonoid exudation occurred at the beginning of cluster-root development, especially in juvenile and immature cluster roots (Fig. 4b). At the mature stage, exudation decreased and was further reduced at the senescent stage.

In order to obtain a more quantitative idea of isoflavonoid concentrations and exudation, we determined the amounts of the 12 major compounds present in and exuded from the four stages of cluster roots. Overall, isoflavonoid concentrations and exudation varied significantly depending on cluster-root stage (ANOVA, $P < 0.05$). Among the 12 major compounds analysed, six showed an altered pattern in concentration and six in exudation level. Figure 5 shows the pattern for four of these compounds. Although, in the chromatogram (Fig. 4), genistein 6''-*O*-malonyl-*O*-glucoside (peak 10; retention time (RT) = 21) displayed higher absorbance than genistein (peak 12; RT = 27.5), the quantification based on calibration curves of purified compounds showed that genistein was the compound produced and exuded in the greatest amounts (Fig. 5a). Genistein (Fig. 5a) did not show any significant change in the pattern of exudation or in internal concentrations. For genistein 7-*O*-diglucoside (Fig. 5b), internal concentrations and exudation showed the same pattern: 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 was observed for genistein 6'-*O*-

malonyl-diglucoside in internal concentrations (Fig. 5c), exudation was higher for juvenile and immature ($P < 0.05$) than for mature and senescent cluster roots. In contrast to genistein 7-*O*-diglucoside and genistein 6'-*O*-malonyl-diglucoside, genistein 6'-*O*-malonyl-*O*-glucoside (Fig. 5d) showed no difference in concentration between the immature and the mature stages, whereas there was a significant decrease in exudation between these two stages. For these four major compounds, isoflavonoid exudation was higher at the juvenile and immature stages, and then decreased at the mature and senescent stages. We calculated the ratios of exudation vs internal concentrations (Fig. 5e) in order to determine whether or not this decrease in isoflavonoid exudation was a consequence of decreased production. In general, ratios varied significantly with cluster-root stage and genistein was exuded in greater amounts than the other three compounds (ANOVA, $P < 0.05$). For all peaks, the ratio was higher (more exudation) in juvenile and immature cluster roots and lower at the mature and senescent stages, suggesting that the decrease in exudation cannot be explained solely by the decrease in internal concentration. 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 (peak 10). No changes were observed between the mature and the senescent stages.

Discussion

In order to obtain new insights into the exudation physiology of cluster roots, we applied a LC-MS approach to characterize the pattern of isoflavonoids produced and exuded at different stages of white lupin cluster roots. We investigated the effects of phosphate supply, root type and cluster-root stage on the quantity and quality of isoflavonoid concentrations and exudation.

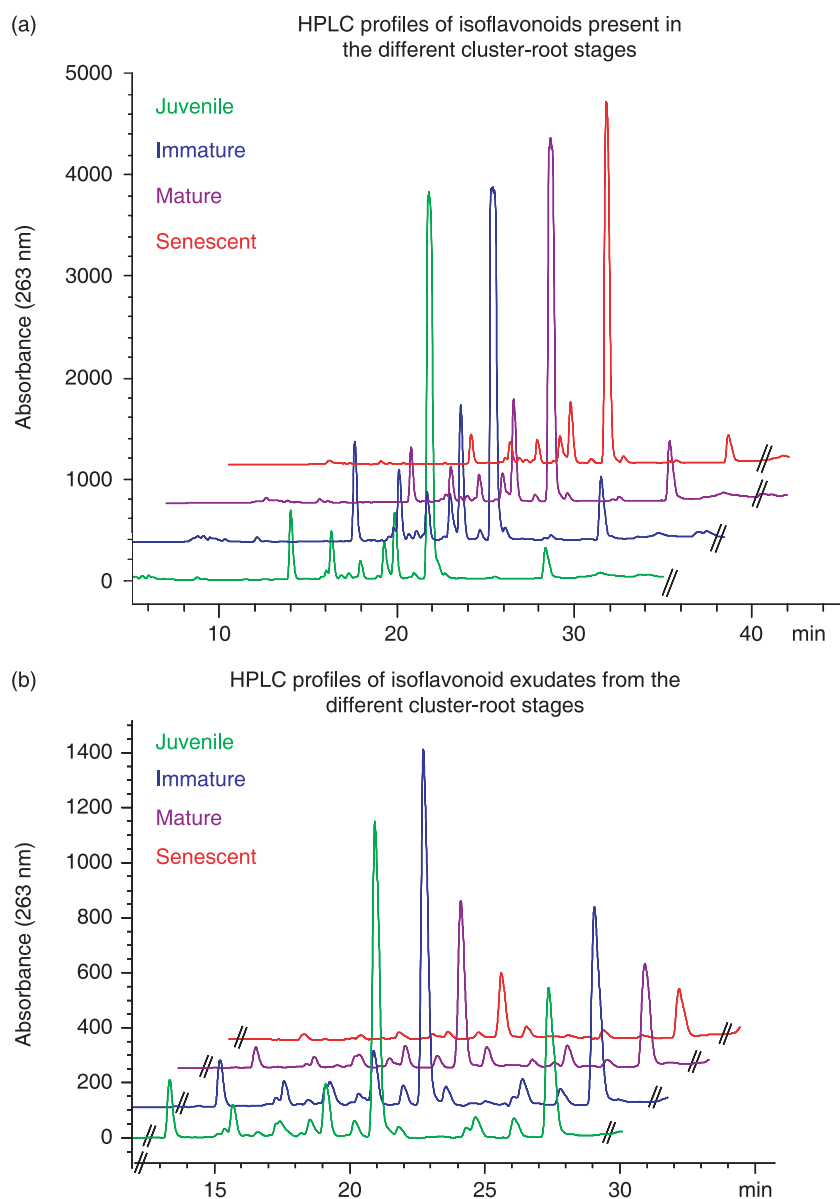


Fig. 4 Effect of cluster-root stage on isoflavonoid concentrations (a) and exudation (b). White lupins (*Lupinus albus*) were grown under phosphorus (P)-deficient conditions. After immersion in a pH indicator solution (see Materials and Methods for more details), cluster roots were separated into four developmental stages: juvenile (green), immature (blue), mature (violet) and senescent (red). Root tissues (concentrations, a) and exudates (exudation, b) were extracted with 80% methanol. Samples were separated on a C18 high-performance liquid chromatography (HPLC) column and absorbance was measured at 263 nm. For internal concentrations (a), 50 μ l was injected, corresponding to 35 mg of roots [fresh weight (FW)]. For exudation (b), 50 μ l was injected, corresponding to a 1-h exudation of 70 mg of roots (FW).

Major isoflavonoids found in white lupin cluster roots

LC-UV-MS techniques were used for structural elucidation and profiling of flavonoid glycosides in root exudates from white lupin. Four diglycosides, six monoglycosides and two aglycones were identified in the extracts (Fig. 1, Table 1). All recognized compounds have been reported previously 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'-hydroxyisulupinalbigenin) from white lupin roots and Bednarek *et al.* (2001) reported the presence of two monoprenylated isoflavonoids, wighteone and luteone. As the plant growth conditions, the extraction method and the profiling analysis were similar in the cited studies and the present work, a possible reason for the differences in the pattern of isoflavonoids recovered might be the fact that different lupin cultivars were used (*L. albus* cv. Bac by Bednarek *et al.* and *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 were detected on the basis of the m/z Y_0^- ions, and the major compounds were related to genistein, while the hydroxygenistein conjugates were less abundant.

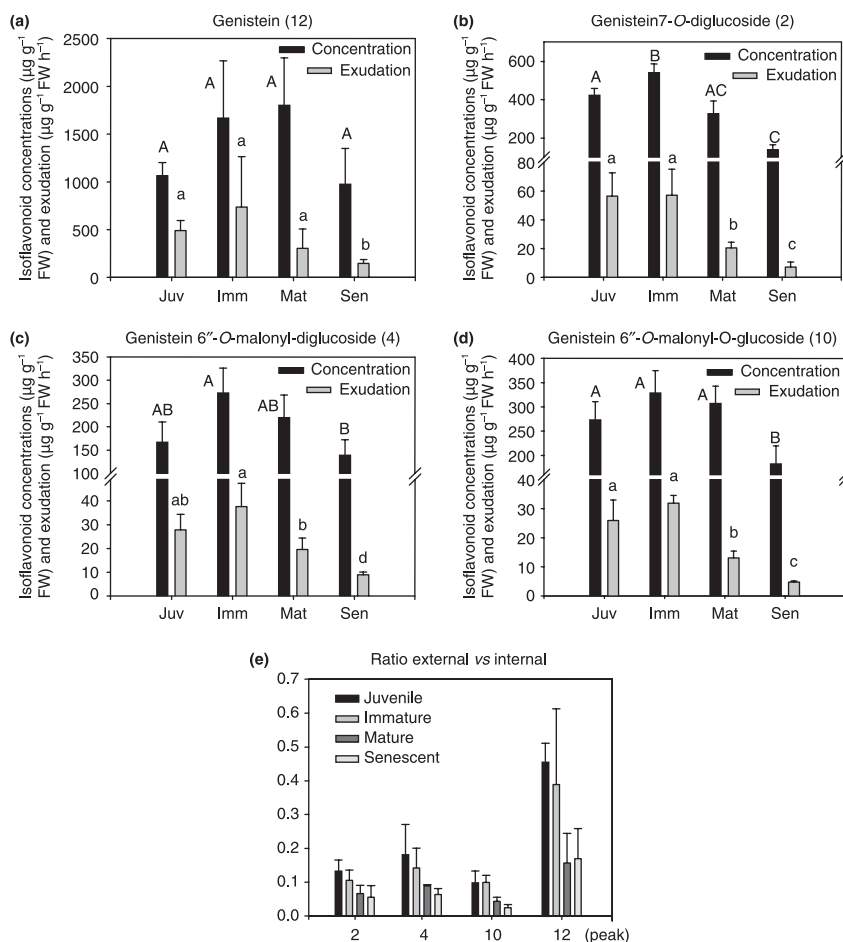


Fig. 5 Quantification (a–d) and external vs internal ratio (e) of four major isoflavonoids in roots and exudates of white lupin (*Lupinus albus*) as a function of cluster-root stage. (a–d) Black bars show the internal concentrations and grey bars show exudation for (a) genistein 7-O-diglucoside, (b) genistein 6'-O-malonyl-diglucoside, (c) genistein 6'-O-malonyl-O-glucoside and (d) genistein. Juv, juvenile; Imm, immature; Mat, mature; Sen, senescent. Bars represent means of three replicates. Different letters (uppercase, internal concentrations; lowercase, exuded amounts) indicate statistically different values (Student's *t*-test, $P < 0.05$). (e) Ratio of exudation vs internal concentrations of four major isoflavonoids as a function of cluster-root stage. Cluster roots were separated into four developmental stages: juvenile (black), immature (grey), mature (dark grey) and senescent (white). peak 2, genistein 7-O-diglucoside; peak 4, genistein 6'-O-malonyl-diglucoside; peak 10, genistein 6'-O-malonyl-O-glucoside; peak 12, genistein. Bars represent means of three replicates.

Effect of P supply

As a lack of phosphate induces the formation of cluster roots in white lupin, we aimed to assess if the pattern of isoflavonoids would differ in plants grown in P-deficient vs P-sufficient conditions. We found that phosphate deficiency caused a general increase in the concentrations and exudation of isoflavonoids (Fig. 2). Although anthocyanin accumulation is a well-known symptom of phosphate deficiency, the effect of phosphate supply on the production and exudation of isoflavonoids at the root level has not often been investigated. To our knowledge, only three studies reported enhanced phenolic production in plants subjected to phosphate deficiency: Murali & Teramura (1985) in soybean plants (*Glycine max*), Juszczuk *et al.* (2004) in bean (*Phaseolus vulgaris*) and Akiyama *et al.* (2002) in melon (*Cucumis melo*). Juszczuk *et al.* (2004) found that, in bean, phosphate deficiency increased the activity of L-phenylalanine ammonia lyase, and also root exudation of phenolics. However, the chemical nature of the exuded phenolic compounds was not investigated. In the case of nitrogen deficiency, the role of isoflavonoids in the signalling leading to symbiosis with

nitrogen-fixing bacteria is well documented and one cannot exclude the possibility that similar compounds might be involved in other kinds of nutrient-deficiency signalling pathways. Supporting this hypothesis, Akiyama *et al.* (2002) found that, in melon roots, P deficiency induced the exudation of a glycosylflavonoid, which is involved in the regulation of the association with arbuscular mycorrhizal fungi. This may indicate that flavonoids, and potentially also isoflavonoids, might be involved in P-deficiency signalling as well as in nitrogen-deficiency signalling.

Differences between cluster and noncluster roots

In comparing the HPLC profiles of cluster and noncluster roots grown in P-deficient conditions (Fig. 3a,c), we found that the profiles were very similar and only differed in the amounts of some isoflavonoids, such as genistein, which was more abundant in cluster roots. Quantitative differences between cluster and noncluster roots were more pronounced for exudation than for internal concentrations, and this suggests that both types of roots are producing the same pattern of phenolic compounds, but that cluster roots are

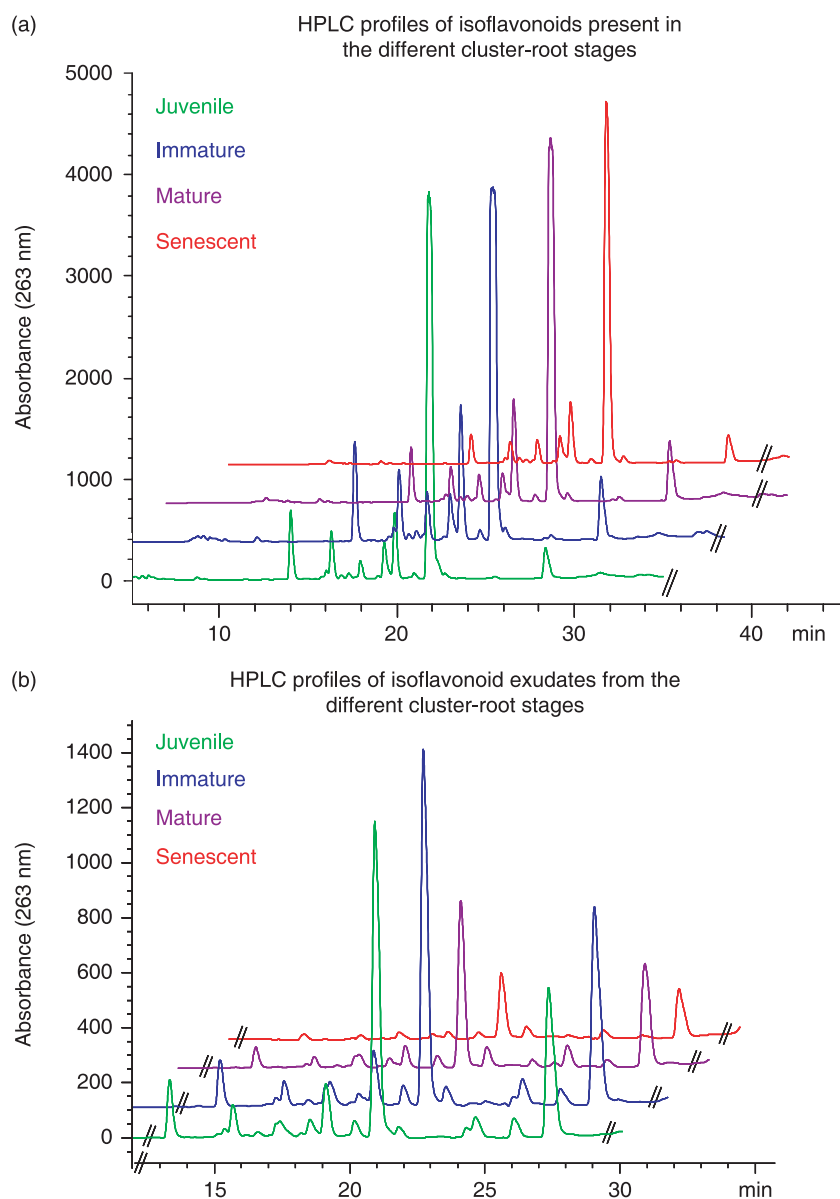


Fig. 4 Effect of cluster-root stage on isoflavonoid concentrations (a) and exudation (b). White lupins (*Lupinus albus*) were grown under phosphorus (P)-deficient conditions. After immersion in a pH indicator solution (see Materials and Methods for more details), cluster roots were separated into four developmental stages: juvenile (green), immature (blue), mature (violet) and senescent (red). Root tissues (concentrations, a) and exudates (exudation, b) were extracted with 80% methanol. Samples were separated on a C18 high-performance liquid chromatography (HPLC) column and absorbance was measured at 263 nm. For internal concentrations (a), 50 μ l was injected, corresponding to 35 mg of roots [fresh weight (FW)]. For exudation (b), 50 μ l was injected, corresponding to a 1-h exudation of 70 mg of roots (FW).

Major isoflavonoids found in white lupin cluster roots

LC-UV-MS techniques were used for structural elucidation and profiling of flavonoid glycosides in root exudates from white lupin. Four diglycosides, six monoglycosides and two aglycones were identified in the extracts (Fig. 1, Table 1). All recognized compounds have been reported previously 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'-hydroxyisoluminalbigenin) from white lupin roots and Bednarek *et al.* (2001) reported the presence of two monoprenylated isoflavonoids, wightone and luteone. As the plant growth conditions, the extraction method and the profiling analysis were similar in the cited studies and the present work, a possible reason for the differences in the pattern of isoflavonoids recovered might be the fact that different lupin cultivars were used (*L. albus* cv. Bac by Bednarek *et al.* and *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 were detected on the basis of the m/z Y_0^- ions, and the major compounds were related to genistein, while the hydroxygenistein conjugates were less abundant.

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Supplementary Material

The following material is available for this article online:

Fig. S1 Electrospray ionization (ESI) mass spectra for O-Glc-Mal-genistein: (a) positive ionization mode [M+H]⁺ ion at mass to charge ratio (m/z) 519; (b) negative ionization mode Y₀⁻ fragment ion at m/z 269 after cleavage of glycosidic bonds. Glc, glucose; Mal, malonyl.

Fig. S2 Nomenclature and diagnostic fragmentation of O-Mal-Glc-genistein. Glc, glucose; Mal, malonyl.