Plant Physiol. (1992) 100, 444–450
0032-0889/92/100/0444/07/$01.00/0

Received for publication February 24, 1992
Accepted April 27, 1992

Localized Changes in Flavonoid Biosynthesis in Roots of *Lotus pedunculatus* after Infection by *Rhizobium loti*[1]

James E. Cooper* and J. Raghavendra Rao

Department of Food and Agricultural Microbiology, The Queen's University of Belfast, Newforge Lane, Belfast BT9 5PX, Northern Ireland, United Kingdom

ABSTRACT

Two-dimensional paper chromatography in four solvent systems, high-sensitivity spray reagents, and UV absorption spectroscopy were used to separate and characterize flavonoids and isoflavonoids in roots and root nodules of 20-d-old *Lotus pedunculatus* Cav. Seedlings were grown either under sterile conditions or after inoculation with Fix$^+$ or Fix$^-$ strains of *Rhizobium loti*. Flavonoids rather than isoflavonoids predominated in all tissues. Flavonoid profiles in sterile and denodulated root tissues were remarkably similar, both qualitatively and quantitatively. At least 14 partially purified flavonoid aglycones and conjugates were found in root extracts; denodulated root tissues contained no compounds that were not also present in sterile roots. Fix$^+$ rhizobia were responsible for major postinfection shifts in plant flavonoid biosynthesis at the sites of nodule morphogenesis. Polymeric flavolans were absent from Fix$^+$ nodules but present in all root tissues and in Fix$^-$ nodules. Catechin was detected only in Fix$^+$ nodules.

Phenolic secondary metabolites are synthesized in both roots and shoot tissues of legumes via the phenylpropanoid/shikimic acid pathways (9). Among these metabolites, the flavonoids are polyphenolic compounds resulting from the expression of two key enzymes: phenylalanine ammonia lyase and chalcone synthase. Flavonoid subgroups such as chalcones, flavones, flavanones, aurones, and isoflavonoids occur in most legume tissues (30) and, together with other aromatic compounds, can be exuded into the rhizosphere. Specific flavonoids found in legume root exudates continue to arouse intense interest due to their role as signal molecules regulating either the induction or the inhibition of nodulation genes in several *Rhizobium* species (16, 17, 24). Additionally, their role as chemoattractants under laboratory conditions has been established (1). These functions, together with other plant-bacterial signal mechanisms involving flavonoids, have been reviewed by Peters and Verma (18).

The influence on the symbiosis of phenolic compounds located inside nodule and root tissue of infected legumes remains unclear. One study (27) has suggested that flavonoids synthesized in alfalfa roots may regulate nodule morphogenesis, possibly via interactions with IAA and leghemoglobin. The isoflavonoid glyceollin is known to accumulate in soybean roots in response to infection by ineffective rhizobia and has been implicated in peribacteroid membrane instability (29). Accumulation of polymeric flavolans (condensed tannins) was reported to be higher in the roots and nodules of ineffectively nodulated *Lotus pedunculatus* plants (14). Pankhurst and Jones (15) further suggested that ineffectiveness may be a consequence of a *Rhizobium* strain's sensitivity to polymeric flavolans during the early stages of infection thread formation. However, studies on a large group of *Lotus* rhizobia (19) indicated that some strains were highly effective on a host plant, while exhibiting sensitivity to its root flavolans in the free-living phase.

Increased biosynthesis of plant phenolics is generally associated with defense responses to physiological stress factors such as light, growth regulators, nutritional deficiencies, wounding, and infection with various microorganisms (25). Infection with *Rhizobium* in particular has been reported to increase phenylalanine ammonia lyase and chalcone synthase activities in soybean plants (5) and methyltransferase activities in bean plants (22). In view of the close connection between synthesis and release of flavonoids in legume roots (13), any changes in flavonoid biosynthesis occurring as a consequence of *Rhizobium* challenge could be expected to influence the synchronized communication between host and microbial cells during the establishment of the symbiosis. However, the timing of such changes in relation to *Rhizobium* infection and the extent to which they alter the types of flavonoid compounds occurring in roots have yet to be clarified. One study (3) reported a stimulatory effect of inoculation on root isoflavonoid concentrations after 12 d in some soybean lines but not in others, whereas another (26) found that *Bradyrhizobium japonicum* caused no changes in the *nod* gene-inducing activity of soybean root extracts during a 3-d postinoculation period.

In this study, we have examined the distribution of the major flavonoids in roots and root nodules of *Lotus pedunculatus* Cav during an 18-d period after inoculation with effective or ineffective rhizobia. These data have been compared with those obtained from the roots of uninoculated plants over the same period of time.

MATERIALS AND METHODS

Bacterial Strains

Detailed results are presented for an effective (Nod$^+$ Fix$^+$) (NZP2037) and an ineffective (Nod$^+$ Fix$^-$) (LC3) strain of *Rhizobium loti*. Experiments were repeated with three other

[1] This work was supported by grants from the Agricultural and Food Research Council, the British Council, and the Nuffield Foundation.

effective (LP1, CC814s, LP24) and ineffective (LC5, LC6, NZP2085) strains of *Lotus* rhizobia. Rhizobia were grown in yeast extract-mannitol broth (28) at 25°C and examined for their colony characteristics on solid media, growth rates in liquid medium, and reaction in unbuffered yeast extract-mannitol agar incorporating a pH indicator. The nitrogen-fixing effectiveness of strains was determined by the method of Cooper (4). Viable counts were obtained routinely by spread-plate culture on yeast extract-mannitol agar (28), and inocula were generated from single colonies on this medium.

Plant Species

Seeds of *Lotus pedunculatus* Cav (Marsh Trefoil, var Grasslands Maku G4705), obtained from the Department of Scientific and Industrial Research, Palmerston North, New Zealand, were surface-sterilized with methanol for 30 s followed by 0.2% mercuric chloride (3 min) and rinsed six times in sterile water to remove traces of sterilant.

To obtain large quantities of root and nodule material for the extraction of flavonoids, plants were grown in anodized aluminum troughs (16 standard wire guage, 44 mm long, 225 mm wide, and 130 mm deep) under aseptic conditions. The troughs were filled to a depth of 100 mm with sand, presterilized in an autoclave at 121°C/15 min, and further sterilized intermittently in a hot air oven at 190°C for 3 h on three consecutive days. The sand was finally covered to a depth of 25 mm with perlite (autoclaved separately at 121°C/15 min). The sand/perlite base was moistened with sterile, N-free rooting solution (4) and adjusted to pH 6.8 to give a semi-soft bed.

Seeds of *L. pedunculatus* were transferred aseptically onto the surface of the perlite/sand bed and allowed to germinate. Groups of 2-d-old seedlings established in this way were inoculated separately with the *R. loti* strains so that the final concentration was 10^3 cells mL^{-1} of rooting solution, and sufficient troughs were prepared to permit duplicate analyses of each treatment at each time interval. Uninoculated troughs were also prepared as controls and sampled in the same way. Plants were grown in a controlled environment chamber with a 17 h day/7 h night at 25°C and a light intensity of approximately 550 μmol photons m^{-2} s^{-1}.

Preparation of Root and Nodule Tissues for Extraction of Flavonoids

Roots and nodules were harvested, washed, and separated 3, 7, 10, 15, and 18 d after inoculation. For each treatment, duplicate 5- to 15-g (fresh weight) samples were used for preparing extracts. The extraction was performed in two stages by macerating the root or nodule tissues first with methanol:water (9:1, v/v) followed by methanol:water (1:1, v/v) containing 1% concentrated HCl (to ensure extraction of anthocyanidin and other low-polarity flavonoids). At each stage, the slurry was filtered to separate the extract from plant material. These two extracts were combined and evaporated under reduced pressure to remove methanol. The aqueous extract was cleared of low-polarity contaminants (e.g. pigments, fats, waxes) by extracting twice with equal volumes of hexane in a separating funnel. The solvent-extracted aqueous layer containing the bulk of flavonoids was concentrated under vacuum in a rotary evaporator. The residue was redissolved in 30% aqueous methanol.

Separation of Flavonoid Constituents by PC

Preliminary analysis of tissue extracts for the main classes of flavonoids was accomplished by PC,[2] and the procedures followed were those described by Markham (12). Separation of individual constituents was achieved by 2D-PC in the descending mode with sheets of Whatman 3MM chromatography paper (460 × 570 mm). Initially, a sample of the root or nodule extract equal to 50 to 100 mg of tissue was spotted on the paper. Four separate chromatograms for each sample were run in the first direction in the following solvent systems in glass chromatotanks (Shandon):

Solvent System A

Butanol:acetic acid:water (4:1:5, v/v) was mixed thoroughly in a separatory funnel. The upper phase was transferred to the tank and equilibrated overnight before starting the PC run. The run took 13 to 17 h for the solvent to reach the lower end of the sheet. This system gave satisfactory resolution of flavonoids into either their glycosides or aglycones.

Solvent System B

Acetic acid:water:hydrochloric acid (30:10:3, v/v) was equilibrated overnight prior to use in separating the phenolics into flavones, flavonols, and anthocyanidin aglycones. Run times were from 9 to 11.5 h.

Solvent Systems C and D

System C (butanol:acetic acid or hydrochloric acid, 1:1, v/v) and system D (tertiary butyl alcohol:acetic acid:water, 3:1:1, v/v) were used to confirm the presence of monomeric flavolans. The solvent system C or D was mixed thoroughly in a separatory funnel and was freshly prepared and equilibrated overnight in the tank prior to running the chromatograms. It was also necessary to equilibrate the chromatogram itself in the solvent vapor prior to starting the run. The run times in these solvents were from 16 to 22 h.

After the run in the first direction, the chromatograms were removed and air-dried in a fume cupboard. They were then run in the second dimension (at right angles to the original direction) for 3.5 h in another tank containing 15% acetic acid (v/v).

Characterization of Flavonoid Compounds after Chromatographic Separation

Compounds were recognized by the position of spots on each 2D-paper chromatogram and provisionally characterized as to class of flavonoid by the colors produced under UV light (366 nm) in the presence or absence of ammonia

[2] Abbreviations: PC, paper chromatography; 2D, two-dimensional; d.a.i., days after inoculation.

vapor (Table I). The spots were carefully outlined with a pencil and the R_F values were determined. These were then compared with the R_F data for flavonoids compiled by Mabry et al. (11). Standard compounds (e.g. apigenin, luteolin, kaempferol, quercetin, catechin, and naringenin supplied by Apin Chemicals, Abingdon, UK) were co-chromatographed under the same conditions, and their R_F values were also used for authentic comparisons. High-sensitivity spray reagents (e.g. 5% $AlCl_3$, 5% vanillin in ethanol/concentrated HCl [4:1, v/v], 1% diphenyl boric acid ethanol-amine complex in methanol) recommended by Markham (12) also were employed to determine spot colors for the main classes of flavonoids (Table I). Further characterization of compounds was achieved by spectrophotometric analyses of liquid chromatogram extracts obtained as described below. UV absorption spectra in MeOH and spectral shifts with $AlCl_3$/HCl were determined with a Gilford UV-vis 2600 spectrophotometer with a 1-cm path length cell.

Recovery and Quantification of the Major Flavonoids Isolated from Root and Nodule Extracts

For recovering the individual flavonoid components in the fresh root or nodule tissues of 20-d-old seedlings, the entire concentrated volumes of the final methanolic extracts were subdivided into aliquots equivalent to 75 mg of fresh tissue and spotted to extinction in multiple aliquots on Whatman 3MM chromatography paper, followed by 2D-PC as described previously. The developed chromatograms were viewed under UV light (366 nm) to locate the spots, which were marked according to specific flavonoid R_F values obtained from earlier runs. Excised spots from a number of identical chromatograms were pooled and soaked in 50 mL of methanol. After 6 to 8 h, during which the contents were gently shaken, the liquid extracts containing the flavonoids were decanted, filtered, concentrated, and purified through a Sephadex LH20 column eluted with 30% aqueous methanol and finally made up to 25 mL.

These solutions were scanned for absorbance peaks (A_{max}) in a Gilford UV-vis 2600 spectrophotometer. To calculate a semi-quantitative expression for the concentrations of the major flavonoids, the Lambert-Beer formula was modified as follows:

$$\text{concentration of flavonoid (mg g}^{-1}\text{ fresh tissue)} = \frac{(ODU \times 1000 \times MW)}{(E \times l \times F),}$$

where *ODU* (optical density units) = absorbance (at A_{max} nm for the flavonoid) × 25 mL; 1000 = factor for conversion to mg; *MW* = mol wt of the flavonoid; l = path length of the cell (d = 1 cm); F = fresh weight (g) of the root or nodule tissue used for the extraction of the flavonoid; and E = extinction coefficient, obtained from the compilations of Jurd (10).

Two types of flavolans were distinguishable: flavolan monomers were quantified from their UV-absorption properties; polymeric flavolans (condensed tannins) were characteristically immobile and remained at the spot of origin. Their concentrations, however, were estimated by the procedures described by Broadhurst and Jones (2) using vanillin-HCl reagent on a 1.0-g portion of fresh root or nodule tissue extracted for polymeric flavolans. Standard curves were obtained after making suitable provision for anthocyanin corrections, and concentrations were calculated.

RESULTS

Data indicating the presence or absence of the main classes of flavonoids in roots and nodules at four sampling times after inoculation are shown in Table II. In general, the types of flavonoids detected during this period were in accordance with conventional plant flavonoid biosynthetic branching patterns. Four major categories of compounds were present: (a) principal precursors (chalcones, flavanones); (b) central precursors (dihydroflavonols); (c) intermediates (flavones, flavonols, aurones, isoflavonoids, monomeric flavolans); and (d) end products (polymeric flavolans).

The roots of sterile plants and of those nodulated by Fix^+

Table I. *Spot Colors of Main Categories of Flavonoids on Paper Chromatograms Treated with Ammonia Vapor or High-Sensitivity Spray Reagents*

Flavonoid Category	Spot Color in UV (365 nm) Light		Spot Color with Spray Reagents for High-Sensitivity Detection		
	Without ammonia vapor	With ammonia	5% $AlCl_3$	Diphenyl-boric acid-ethanolamine complex	Vanillin-HCl reagent
4, 5/4 hydroxy flavones/flavonols	Dark purple	Yellow, green tinge	Yellow, fluorescent under UV (365) light	Orange	Purple
Flavanones, chalcones, dihydroflavonols	Dark purple	No change	Yellow	Deep orange	Red (after warming)
Isoflavones	Invisible	Fluorescent blue	Fluorescent blue (under UV light)	Mauve/pink	Red
Aurones or chalcones	Fluorescent yellow, green	Deep red, rapidly fading to orange	Fluorescent yellow (under UV light)	Mild green	Purple
Catechins, proanthocyanidins	Invisible/nonfluorescent (mobile)	No change	Yellow tinge	Mild, fading to orange	Deep red to dark purple
Polymeric products	Invisible/nonfluorescent (at the spot of origin)	No change	No reaction	Deep brown	Red to purple

Table II. *Major Categories of Flavonoids in L. pedunculatus Roots and Root Nodules*

Tissue Sample	Sampling Time (d after inoculation)																			
	Principal Precursors (chalcones, flavanones)				Central Precursors (dihydroflavonols)				Intermediates (flavones, flavonols, aurones, isoflavonoids)				Intermediates (monomeric flavolans)				End products (polymeric flavolans)			
	3	7	10	15	3	7	10	15	3	7	10	15	3	7	10	15	3	7	10	15
Denodulated roots (Fix^+ strain)	+[a]	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	−	+	+
Denodulated roots (Fix^- strain)	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	−	+	+
Sterile roots	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	−	+	+
Nodules (Fix^+ strain)	NS[b]	+	+	+	NS	+	+	+	NS	+	+	+	NS	−	+	+	NS	−	−	−
Nodules (Fix^- strain)	NS	NS	+	+	NS	NS	+	+	NS	NS	+	+	NS	NS	+	+	NS	NS	+	+

[a] +, Present; −, not detected. [b] NS, No sample available.

or Fix^- rhizobia contained the same members of the first two categories of compounds at all sampling times. This was also the case for the intermediate compounds, with the exception of monomeric flavolans, which were detected in all root extracts only at 10 and 15 d.a.i. Polymeric flavolans also were detected in the three types of root extracts at these two sampling times.

Nodules formed by Fix^+ strain NZP2037 were visible, and therefore available for harvesting, at 7 d.a.i. Nodules of Fix^- strain LC3 were not visible until 10 d.a.i. and so they were harvested only at two sampling times. Principal and central precursors and a large group of intermediates were present in both types of nodules at all sampling times. Monomeric flavolans were found in both types of nodules at 10 and 15 d.a.i., whereas polymeric flavolans were detected only in Fix^- nodules. When this experiment was repeated with three more effective and ineffective strains, the same patterns and differences in flavonoid distribution were observed in all cases.

Table III. *Distribution of Flavonoid Compounds in Roots and Root Nodules of L. pedunculatus Seedlings Sampled 18 d after Growth Under Sterile Conditions or Inoculation with R. loti Strains*

Type of Flavonoid	R_F Value with Butanol:Acetic Acid:Water (4:1:5)[a]	Presence of Compounds in Root and Nodule Tissues[b]				
		Sterile (uninoculated) roots	Denodulated roots (Fix^+ strain)	Denodulated roots (Fix^- strain)	Nodules (Fix^+ strain)	Nodules (Fix^- strain)
Dihydroflavonols						
Dihydrokaempferol	0.86	+	+	+	+	+
Dihydrokaempferol-7-glucoside	0.54	+	+	+	−	−
Dihydroquercetin	0.76	+	+	−	+	+
Flavones						
Luteolin	0.75	+	+	+	+	+
Luteolin-5-glucoside	0.82	+	+	+	+	+
Apigenin	0.88	+	+	+	−	−
Flavonols						
Kaempferol	0.85	+	+	+	+	+
Kaempferol-7-rhamnoside	0.72	+	+	+	+	+
Quercetin	0.66	+	+	+	+	+
Quercetin-3-rhamnoglucoside	0.42	+	+	+	+	+
Rhamnetin	0.70	+	−	+	+	−
Monomeric flavolans						
Catechin	0.66	−	−	−	+	−
Delphinidin	0.42	+	+	+	−	+
Polymeric flavolans						
Procyanidin	Immobilized at origin	+	+	+	−	+
Prodelphinidin		+	+	+	−	+
Other flavonoids						
Aurones, chalcones, flavanones, isoflavonoids		+	+	+	+	+

[a] R_F values, UV absorption spectra in methanol, and spectral shifts with $AlCl_3$/HCl were consistent with published standards (11).
[b] +, Present; −, not detected.

A more detailed analysis of the flavonoid content of roots and root nodules at a single sampling time is presented in Table III. This information was obtained from extracts of plant material harvested at 18 d.a.i., and the provisional characterization of individual compounds was based on R_F values from four solvent systems, color characteristics under UV light in the presence and absence of ammonia vapor, color reactions with a range of high-sensitivity spray reagents, UV spectral analysis of methanol extracts, and spectral shifts with $AlCl_3$/HCl.

Distribution patterns for uninoculated and both types of denodulated root tissue were remarkably similar. Among the central precursors and intermediates, some compounds were detected in conjugated and aglycone forms. Dihydroflavonols were found in all roots, but dihydroquercetin was not detected in the roots of plants nodulated by the Fix^- strain. Among the intermediates, rhamnetin was the only compound not present in all types of root tissue.

The monomeric flavolans in roots were represented by a single compound, delphinidin, which was present in all samples. Polymeric flavolans were present in all root samples in the forms of procyanidin and prodelphinidin. Other flavonoids comprising principal precursors (chalcones, flavanones) and intermediates (aurones, isoflavonoids) also were detected in all types of root tissue.

Fix^+ and Fix^- nodules also displayed few differences in their range of flavonoid precursors and intermediates; rhamnetin was the only compound in these categories to be detected in one type of nodule. Certain compounds present in root samples (dihydrokaempferol-7-glucoside, apigenin, rhamnetin [Fix^- samples only]) were not detected in the corresponding nodule tissue, whereas two compounds present in nodules were undetectable in host root tissue (dihydroquercetin [Fix^- samples only] and rhamnetin [Fix^+ samples only]).

The differences between Fix^+ and Fix^- nodules were substantial with regard to monomeric and polymeric flavolans. Catechin was present in Fix^+ but not in Fix^- nodules, whereas the converse was true for delphinidin. Polymeric flavolans were absent from Fix^+ nodules, but Fix^- nodules contained these end products in two forms: procyanidin and prodelphinidin.

Quantitative data for various groups of flavonoids in tissues harvested 18 d.a.i. are presented in Figure 1. In the three types of root tissue, amounts of precursors and intermediates were similar, but larger quantities of polymeric flavolans were found in roots nodulated by the Fix^- strain. In root tissues, the largest contribution (>80%) to the nonflavolan intermediates pool was made by flavonols and flavones with only a minor presence of aurones and isoflavonoids. There were marked differences in both the qualitative and quantitative distribution of flavonoids in Fix^+ and Fix^- nodules (Fig. 1). In Fix^+ nodules, flavones and flavonols formed a high proportion (approximately 70%) of the total flavonoid pool. Monomeric flavolans were present in amounts similar to those in root tissue, but polymeric flavolans (condensed tannins) were absent. In marked contrast, flavone and flavonol intermediates were present in much smaller quantities in Fix^- nodules (about 15% of the total flavonoid pool), whereas monomeric and polymeric flavolans were found in large amounts. In both types of nodule, as in root tissues, aurones and isoflavonoids made only a small contribution to the pool of nonflavolan intermediates.

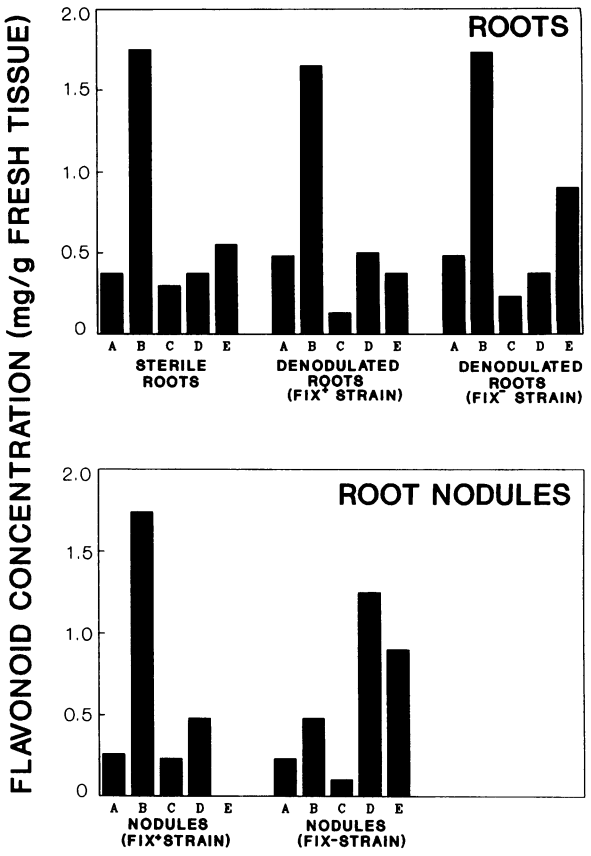

Figure 1. Concentration of flavonoid precursors, intermediates, and end products in roots and root nodules of *L. pedunculatus* 18 d after inoculation: A, Principal and central precursors (chalcones, flavanones, dihydroflavonols); B, intermediates (flavones, flavonols); C, intermediates (aurones, isoflavonoids); D, intermediates (monomeric flavolans); E, end products (polymeric flavolans).

DISCUSSION

Several results from this study point to the fact that Fix^+ rhizobia in particular are responsible for major shifts in the flavonoid biosynthetic pathways of plant cells. First, catechin, which is not synthesized in *L. pedunculatus* roots (15), was detected only in Fix^+ root nodules and is the first new product of flavonoid biosynthesis to be identified in this legume following *Rhizobium* infection. Second, apigenin, which was detected in adjacent root tissue, was not present in nodules. Third, polymeric flavolans were not found in Fix^+ root nodules, although they were present in sterile and denodulated root samples and also in Fix^- root nodules.

Our results also suggest an influence of *Rhizobium* on sets of genes (6) that coordinate the formation of flavonoids downstream from chalcones. The proposed effects of rhizobia on various stages of flavonoid biosynthesis in roots and developing root nodules are described in Figure 2. It is clear that the main emphasis of biosynthesis was toward flavo-

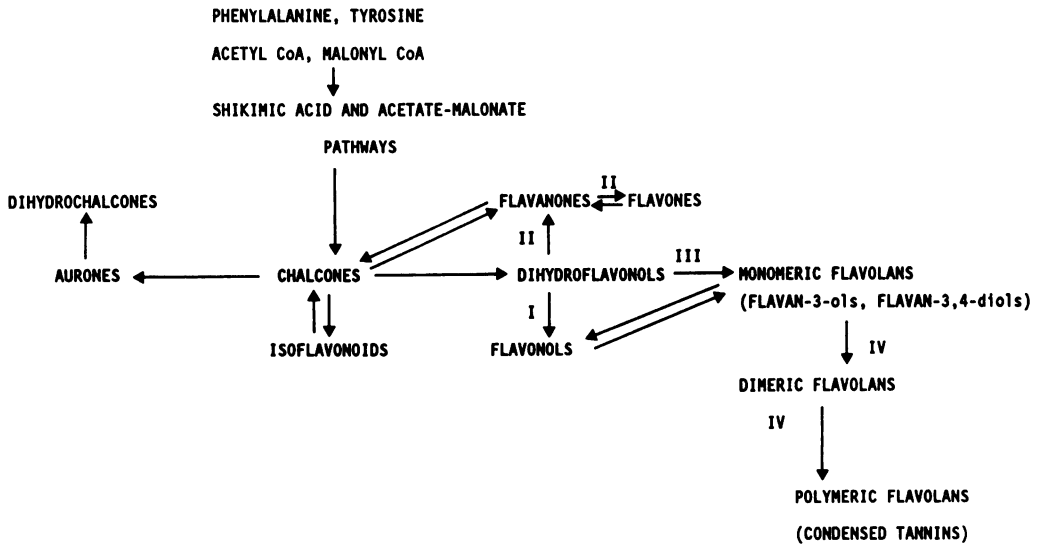

Figure 2. Flavonoid biosynthetic pathways in roots and root nodules of *L. pedunculatus* seedlings. For explanation of symbols see the text. Based on the scheme of Heller and Forkmann (9).

noids rather than isoflavonoids. This contrasts sharply with the situation in young soybean roots, where isoflavonoids are the major products from chalcones (7).

Routes I and II (formation of flavonols and flavones, respectively, Fig. 2) were active during the early stages of root nodule development (0–10 d.a.i.), whereas routes III and IV (formation of monomeric and polymeric flavolans, respectively, Fig. 2) appear to be active in a tissue-specific fashion from about 10 d.a.i. In uninfected root tissues, routes I to IV were operating simultaneously, whereas in Fix^- nodules the major products were flavolans (routes III and IV) at the expense of routes I and II. In Fix^+ nodules, route IV was not operative. The timing of these events is such that rhizobia would not encounter polymeric flavolans during infection and nodule development; therefore, it seems unlikely that these toxic condensates would have any causative influence on the effectiveness of the microsymbiont.

It has been suggested that de novo biosynthesis inside the root could be the source of new *nod* gene-inducing flavonoids that have been found in the root exudates of a legume following exposure to *Rhizobium* (21, 23). However, the new compounds have not been detected in root tissues, and our own results provide no support for this theory. At least 14 partially purified flavonoid aglycones and conjugates were found in the *L. pedunculatus* root extracts, but denodulated roots contained no compounds that were not also present in sterile roots. Studies of 3-d-old sterile seedlings of alfalfa by Maxwell and Phillips (13) showed that synthesis and release of flavonoids by roots occur concurrently, and any compounds secreted into the root exudate should be detectable in root extracts either as aglycones or conjugates. Our data indicate that flavonoid biosynthesis is unaffected in those parts of the root that are merely subjected to remote signaling from, or to surface contact with, rhizobia. The similarity of flavonoid profiles in sterile and denodulated root tissue and the detection of major profile changes in root nodules point to the conclusion that the influence of *Rhizobium* on flavonoid biosynthesis in plant cells is a postinfection process localized at the sites of nodule morphogenesis.

In the absence of firm evidence for a link between the appearance of new flavonoids in the root exudates of inoculated legumes and their biosynthesis in roots, it may be necessary to consider explanations for the presence of these compounds that involve ex vivo chemical or biochemical transformations. For example, under appropriate physiological conditions, spontaneous isomerization of chalcones can yield a mixture of chalcones and flavanones (9). Plant enzymes in the rhizosphere could also be responsible for flavonoid transformations or, as suggested by Graham (7) in the case of the isoflavone diadzein in soybean root exudates, conversion of conjugates to aglycones. Free-living rhizobia themselves could be responsible for changes in the flavonoid content of root exudates, either indirectly through effects of their metabolism on the chemical environment of the rhizosphere (e.g. pH shifts) or directly by reactions such as the hydrolysis of flavone conjugates (8) or the degradation of flavones to yield, among other products, chalcones (20).

The precise relationships between synthesis and release of flavonoids in legume roots, together with any influence of rhizobia on the two processes in the pre- and early infection phases, could be clarified by making detailed comparisons of flavonoid profiles in root extracts and exudates of sterile and inoculated plants. High-resolution HPLC with diode or spectral array detection, capillary zone electrophoresis, and ^{14}C incorporation into flavonoid precursors with subsequent label tracing would be particularly valuable techniques for such studies.

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