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Concurrent Synthesis and Release of *nod*-Gene-Inducing Flavonoids from Alfalfa Roots[1]

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ABSTRACT

Flavonoid signals from alfalfa (*Medicago sativa* L.) induce transcription of nodulation (*nod*) genes in *Rhizobium meliloti*. Alfalfa roots release three major *nod*-gene inducers: 4′,7-dihydroxyflavanone, 4′,7-dihydroxyflavone, and 4,4′-dihydroxy-2′-methoxychalcone. The objective of the present study was to define temporal relationships between synthesis and exudation for those flavonoids. Requirements for concurrent flavonoid biosynthesis were assessed by treating roots of intact alfalfa seedlings with [U-^{14}C]-L-phenylalanine in the presence or absence of the phenylalanine ammonia-lyase inhibitor L-2-aminoxy-3-phenylpropionic acid (AOPP). In the absence of AOPP, each of the three flavonoids in exudates contained ^{14}C. In the presence of AOPP, ^{14}C labeling and release of all the exuded *nod*-gene inducers were reduced significantly. AOPP inhibited labeling and release of the strongest *nod*-gene inducer, methoxychalcone, by more than 90%. Experiments with excised cotyledons, hypocotyls, and roots incubated in solution showed that the flavonoids could be synthesized in and released from each organ. However, the ratio of the three flavonoids in exudates from intact plants was most similar to the ratio recently synthesized and released from excised roots. A portion of recently synthesized flavonoid aglycones was found conjugated, presumably as glycosides, in root extracts and may have been involved in the release process. Data from root extracts showed that formononetin, an isoflavonoid which does not induce *nod* genes, was present in conjugated and aglycone forms but was not released by normal intact roots. In contrast, roots stressed with $CuCl_2$ did release the aglycone formononetin. Thus, the release process responsible for exudation of *nod*-gene inducers appears to be specific rather than a general phenomenon such as a sloughing off of cells during root growth. The synthesis and specific concurrent release of flavonoid *nod*-gene inducers in this study is consistent with the physiological requirement for nodule formation of the 3-day-old seedlings used.

Alfalfa (*Medicago sativa* L.), an important leguminous forage crop, forms N_2-fixing root nodules in association with the soil bacterium *Rhizobium meliloti*. Early events of alfalfa nodule formation require expression of nodulation genes including *nodDABC* on the megaplasmid of *R. meliloti* (6, 15). Transcription of *nodABC* is induced through the cooperative action of the protein product of the *nodD* gene and components of root and seed exudates (21). Luteolin, 3′,4′,5,7-tetrahydroxyflavone, and chrysoeriol, 3′-methoxy-4′,5,7-trihydroxyflavone, are present in alfalfa seed exudates and can induce rhizobial *nod* genes (9, 23). Those compounds are not found in alfalfa root exudates, but other flavonoid *nod*-gene inducers, 4,4′-dihydroxy-2′-methoxychalcone, 4′,7-dihydroxyflavone, and 4′,7-dihydroxyflavanone, are present (20). Related flavonoids can induce *nod*-gene transcription in other *Rhizobium* and *Bradyrhizobium* species (2, 7, 16, 25, 26, 30).

The temporal relationship between synthesis and exudation of flavonoids in roots is poorly understood. One possibility is that flavonoids are synthesized in the previous generation, stored in the seed, and then transported to roots for release during seedling growth. This prospect is hypothetically possible for the dominant alfalfa *nod*-gene inducer, luteolin, because it is present in extracts of unimbibed alfalfa seeds (8, 23). However, with the exception of trace amounts of 4′,7-dihydroxyflavone, no *nod*-gene inducer found in root exudates also has been identified in alfalfa seeds (8) or seed exudates (9). As an alternative to complete synthesis in the previous generation, precursor flavonoids stored in seeds may be modified to *nod*-gene inducers with known reactions (10), transported to roots, and released during imbibition.

A second possibility is that *nod*-gene-inducing flavonoids are synthesized *de novo* during early seedling growth. Such synthesis presumably would require active phenylpropanoid metabolism, including PAL[2] activity, to supply carbon skeletons for general flavonoid formation (10). Newly synthesized flavonoids could be released directly into the rhizosphere or conjugated and stored in vacuoles. In many plants, flavonoids are stored in vacuoles as glycosides or related conjugates (14, 19), while aglycones are the major form secreted (29). Movement from the vacuole to the external environment would require conversion from conjugate to aglycone. Such a process was suggested by D'Arcy-Lameta (3) to occur during release of flavonoids by roots, but little evidence was presented to support that possibility. Many plants have enzymatic activities that hydrolyze flavonoid conjugates (11–13, 24, 27), but a direct involvement of those enzymes in the exudation of *nod*-gene-inducing flavonoids has not been demonstrated. The purpose of this study was to define temporal relationships between synthesis and exudation of *nod*-gene inducers from uninoculated roots of alfalfa seedlings using [U-^{14}C]-L-phen-

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[2] Abbreviations: PAL, L-phenylalanine ammonia-lyase; AOPP, L-2-aminoxy-3-phenylpropionic acid; δ_H, chemical shift of proton; 2H, two protons, etc.; C-1, carbon one, etc.; *d*, doublet; *s*, singlet; *m*, multiplet; *J*, coupling constant.

ylalanine in the presence and absence of the PAL inhibitor AOPP (1).

MATERIALS AND METHODS

Plant Culture

Rhizobium-free alfalfa (*Medicago sativa* L, cv 'Moapa 69') seedlings were grown as previously described (20) in a hydroponic system with sterile N-free nutrient solution (4), an irradiance of 350 to 400 $E \cdot m^{-2} \cdot s^{-1}$ (400–700 nm), 16/8 h light/dark, 25°C/20°C, and 50% RH. Seed imbibition was started 8 h after the growth chamber lights came on. Nutrient solutions were changed 48 h later and at subsequent intervals determined by the experiment.

Chemicals

AOPP was obtained from Molecular Probes, Inc. Formononetin (7-hydroxy-4'-methoxyisoflavone), 4',7-dihydroxyflavanone, and 4',7-dihydroxyflavone were purchased from Spectrum Chemical Mfg. Corp., Gardena, CA, and 4,4'-dihydroxy-2'-methoxychalcone was a gift from Dr. R. E. Carlson, Ecochem Research, Inc. [U-^{14}C]-L-phenylalanine and [U-^{2}H]acetone were purchased from ICN Biomedicals, Inc., and Sigma Chemical Co., respectively.

Intact Seedling Experiments

Two containers, each with about 400 64-h-old alfalfa seedlings, were used in each intact plant experiment. The hydroponic reservoir contained 170 mL of glass beads (4 mm) and 150 mL of nutrient solution. Nutrient solution of one container was supplemented to contain 0.3 mM AOPP, and 2 h later, 80 μCi of [U-^{14}C]-L-phenylalanine (405 μCi/μmol) were placed in each container. When the experiment was repeated, 125 μCi of [U-^{14}C]-L-phenylalanine (344 μCi/μmol) were used. After 8 h of incubation, nutrient solutions were collected, and roots and shoots were harvested separately, weighed, and stored at −80°C. Replicate samples for laboratory analyses were created by subdividing nutrient solutions and plant parts from each treatment into three subsamples. Results from two experiments were pooled using a split-plot statistical analysis.

Excised Organ Experiments

Approximately 15 68-h-old alfalfa seedlings were cut into roots, hypocotyls, and cotyledons. Three replicate samples (150–400 mg fresh weight) from each plant organ were placed in 2.5 mL of nutrient solution with or without 0.3 mM AOPP. After incubating in the growth chamber for 1 h on a shaker (500 rpm), fresh nutrient solutions supplemented to contain 5 μCi of [U-^{14}C]-L-phenylalanine (405 μCi/μmol) with or without AOPP were supplied. After another 4 h of incubation (70–74 h after the start of imbibition), flasks were transferred to ice, plant parts were harvested, rinsed, and stored at −80°C.

$CuCl_2$ Experiment

Alfalfa seedlings were treated with 3.3 mM $CuCl_2$ to increase flavonoid synthesis (5) by supplementing the initial root solution 47 h after seed imbibition began. Solutions of control and $CuCl_2$-treated plants were replaced 1 h later (48 h) with solution that contained no $CuCl_2$. Root solutions were changed every 8 h between 48 and 80 h and saved for analysis. Four replicates, each consisting of two containers, were collected from the two treatments at every sampling time.

Isolation and Purification of Flavonoids

Exudate solutions were supplemented with known quantities of 4',7-dihydroxyflavanone, 4,4'-dihydroxy-2'-methoxychalcone, and 4',7-dihydroxyflavone, centrifuged at 6500*g*, and processed with C_{18} cartridges as previously described (20). Dried acetone eluants of C_{18} cartridges were stored at −80°C, then solubilized in 50% (v/v) methanol in water, and fractionated by analytical HPLC. HPLC separations were achieved with a standard gradient (0.5 $mL \cdot min^{-1}$), which consisted of an isocratic solvent system of 52.5:45:2.5 (v/v/v) water:methanol:acetic acid (0–20 min), a linear gradient to 60:37.5:2.5 (v/v/v) methanol:water:acetic acid (20–30 min), and isocratic separation at the final concentration (30–60 min). HPLC fractions were collected every 30 s and analyzed for radioactivity by liquid scintillation counting.

Fresh plant material from AOPP experiments was ground in liquid N_2, extracted with 80% (v/v) methanol in water, and centrifuged at 9600*g*, 5°C for 20 min. The pellet was reextracted twice with 80% methanol, and all supernatants were passed through a C_{18} cartridge to remove lipid. Eluants dried with N_2 at 45°C were resolubilized in 50% methanol, diluted with water to 30% (v/v) methanol in water, and applied to a C_{18} cartridge. Flavonoid conjugates and aglycones were eluted with 30 and 100% methanol, respectively. Eluants were dried with N_2 at 45°C and stored at −80°C. The conjugate fraction, presumably glycosides (28), was hydrolyzed in 1 mL of 2 M HCl at 100°C for 30 min, cooled, and applied to a C_{18} cartridge. The cartridge was rinsed with 2 mL of water and 6 mL of 30% methanol before aglycones were removed in 100% methanol and dried with N_2 at 45°C. Aglycone fractions were solubilized in 50% methanol and separated by analytical HPLC. HPLC fractions containing 4',7-dihydroxyflavone were collected and dried with N_2 at 45°C. The dried samples were solubilized in 50% methanol and further purified on the HPLC with appropriate methanol:water:acetic acid gradients to separate closely running compounds. HPLC fractions were collected and analyzed for radioactivity by liquid scintillation counting.

Initial surveys testing for the presence of *nod*-gene inducer conjugates in roots used more rigorous precautions to preserve fragile molecules (17). Differences from the above protocol included extraction of 48-h-old roots with cold (<0°C) acetone and drying under N_2 at 15°C or less. No C_{18} cartridges were employed; the complete extract was processed on the analytical HPLC. HPLC fractions were collected every 30 s, combined when associated with absorbance peaks, and assayed for *nod*-gene-inducing activity (below). To test for conjugates

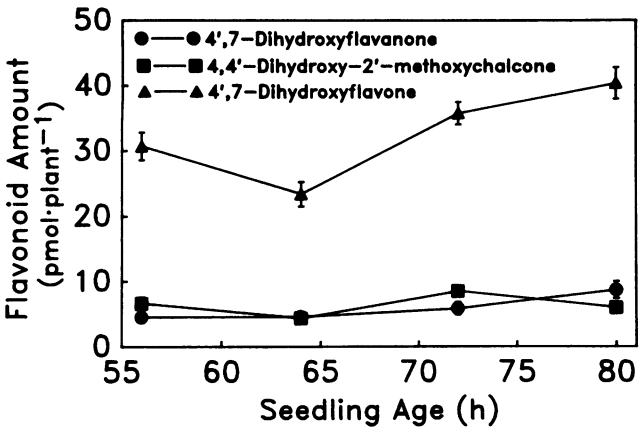

Figure 1. Release of flavonoid *nod*-gene inducers from roots of intact alfalfa seedlings. Seedling age was measured from the start of seed imbibition. Points represent mean ± SE of three replicates.

of *nod*-gene inducers, duplicate samples were hydrolyzed with 2 M HCl at 100°C for 30 min, and aglycones were partitioned from the acid into ethyl acetate. The ethyl acetate fraction was dried at 45°C under N_2, resolubilized with 50% methanol, and analyzed by HPLC.

Identification, Quantification, and Bioassays of Flavonoids

Aglycones previously found in this system (20) were identified in HPLC eluants by retention time and spectral traits. Quantification and bioassays for *nod*-gene-inducing activity with a *nodC-lacZ* fusion on plasmid pRmM57 in *Rhizobium meliloti* strain 1021 were done as described previously (20). All values were corrected for percent recovery as determined for authentic standards.

The predominant flavonoid in alfalfa roots, later recognized as formononetin, was isolated from extracts and identified by UV/visible spectroscopy with shift reagents (18, 20), one-dimensional proton NMR (20), and MS (20). NMR experiments were done with [U-^{2}H]acetone instead of [U-^{2}H]-methanol (20) and were referenced to the methyl peak (2.05 ppm) of acetone. After tentative identification, an authentic standard was obtained, analyzed by each of the above procedures, and tested by cochromatography with the unknown in the analytical HPLC system. The log ϵ value of formononetin, 4.50 at 249 nm in methanol, was determined gravimetrically on a XA-200DS analytical balance (Fisher Scientific Corp.).

RESULTS

Intact Seedling Experiments

Root exudates collected every 8 h from 48- to 80-h-old seedlings showed minor variations in the amounts of 4',7-dihydroxyflavone, 4',7-dihydroxyflavanone, and 4,4'-dihydroxy-2'-methoxychalcone, but the flavone was released in amounts approximately fivefold greater than the latter two compounds (Fig. 1). Because the greatest amount of 4,4'-dihydroxy-2'-methoxychalcone was released between 64 and 72 h, 64- to 68-h-old seedlings were used as starting material for studies of concurrent synthesis and release.

Each of the three flavonoid *nod*-gene inducers collected in root exudates between 66 and 74 h was radiolabeled when ^{14}C-phenylalanine was fed to roots of intact seedlings over that period (Table I). Incorporation of radioactivity into the exuded inducers was decreased 92 to 96% by the PAL inhibitor AOPP. Furthermore, AOPP inhibited total release of those flavonoids by 50 to 93% (Table II). Exudation of the two stronger *nod*-gene inducers, 4,4'-dihydroxy-2'-methoxychalcone and 4',7-dihydroxyflavone, was decreased by AOPP to a greater extent, in both absolute and proportional terms, than that of 4',7-dihydroxyflavanone.

Excised Organ Experiments

The three flavonoid *nod*-gene inducers normally exuded by roots also were radiolabeled and released from hypocotyls and cotyledons when ^{14}C-phenylalanine was fed to those plant organs (Table III). As in the case of roots attached to intact seedlings, treating the organs with AOPP inhibited the incorporation of ^{14}C from phenylalanine (Table III) and decreased total release of the *nod*-gene inducers from the excised organs (Table IV). The ratio of the three flavonoids recently synthesized and released from excised roots was more similar to the ratio in exudates from intact seedlings than corresponding values from other excised plant organs (Tables I *versus* III). Exudation of 4,4'-dihydroxy-2'-methoxychalcone from plant parts was lower, in relation to the other *nod*-gene inducers, than for intact plants (Tables II *versus* IV). Cotyledons released the smallest amount of the chalcone, but difficulties associated with integrating areas under small peaks prevented that quantity from being significantly less than the amount from roots.

Root Extracts

Root extracts from 48-h-old roots contained conjugates of 4',7-dihydroxyflavone and 4',7-dihydroxyflavanone, but no conjugates of 4,4'-dihydroxy-2'-methoxychalcone were detected in any sample (Fig. 2A). The flavone and flavanone aglycones were released by hydrolysis of HPLC fractions with retention times between 9 and 15 min. The greatest amount was released from the 13 to 15 min fraction, peak A. Small amounts of the flavone were also observed after hydrolysis of material eluting between 5 and 9 min. Fourfold more 4',7-dihydroxyflavone conjugate than aglycone was present in root

Table I. *Effects of the PAL Inhibitor AOPP on Incorporation of [U-^{14}C]-L-Phenylalanine into Flavonoids Released from Roots of Intact Alfalfa Seedlings*

Each value represents the mean of three samples.

Treatment	4',7-Dihydroxy-flavanone	4,4'-Dihydroxy-2'-methoxychalcone	4',7-Dihydroxy-flavone
		dpm·g fresh wt^{-1}	
Control	3,130	3,250	24,500
AOPP	80	85	952
$LSD_{(0.05)}$	186	1,330	4,810

Table II. *Effects of the PAL Inhibitor AOPP on the Release of Flavonoids from Roots of Intact Alfalfa Seedlings*

Each value represents the mean of three samples.

Treatment	4′,7-Dihydroxy-flavanone	4,4′-Dihydroxy-2′-methoxychalcone	4′,7-Dihydroxy-flavone
		pmol·g fresh wt^{-1}	
Control	336	547	1,910
AOPP	170	41	206
$LSD_{(0.05)}$	154	269	315

extracts. UV/visible spectral data showed some 4′,7-dihydroxyflavone aglycone was present in peak C, but no 4′,7-dihydroxyflavone aglycone was observed at its expected elution time of 25 min. Following acid hydrolysis, however, traces of the flavanone frequently were detected by UV/visible spectral analyses of the region containing peak F_1. Hydrolysis of region B produced similar evidence for 4,4′-dihydroxy-2′-methoxychalcone.

Many regions of the HPLC chromatogram had *nod*-gene-inducing activity in *R. meliloti* (Fig. 2B). The two most active areas had retention times equal to those of 4,4′-dihydroxy-2′-methoxychalcone, peak B, and 4′,7-dihydroxyflavone, peak C. Although peak C had an absorption spectrum identical to 4′,7-dihydroxyflavone, further chromatography of peak B was required to isolate a compound with the absorption spectrum of the methoxychalcone. The major compound in root extracts, peak F_2, was converted to peaks F_1 and F with mild hydrolysis (30 min, 2 M HCl at 25°C). Stronger hydrolysis (30 min, 2 M HCl at 100°C) produced only peak F. Peak F had the same retention time and UV/visible absorption spectrum as formononetin. UV/visible spectroscopic shift analyses of peak F and authentic formononetin gave equivalent results that were consistent with published data for formononetin (18). Both the putative and authentic formononetin had identical proton resonance patterns in one dimensional proton NMR experiments: δ_H ppm ([U-^{2}H]acetone); 8.18 (1H, *s*, C-2), 8.07 (1H, *d*, J = 8.7 Hz, C-5), 7.56 (2H, *d*, J = 8.3 Hz, C-2′, C-6′), 7.01 to 6.90 (4H, *m*, C-3′, C-5′, C-6, C-8), 3.83 (3H, *s*, -OCH_3). MS analyses of the putative and authentic formononetin showed both had a mol wt of 268 and a major ion fragment of 132. On the basis of those data, it was concluded that peak F was formononetin, which previously was identified in older roots of alfalfa (22).

Formononetin was not exuded normally by roots, but it was released in response to $CuCl_2$ treatment (Fig. 3). Tests for *nod*-gene induction showed that formononetin did not induce transcription over a range of 4 nM to 100 μM (data not shown). The *nod*-gene-inducing activity in peaks containing formononetin and its conjugates (Fig. 2A, peaks F, F_1, F_2) presumably was associated with coeluting compounds.

A detailed examination of ^{14}C incorporation into the conjugate and aglycone forms of 4′,7-dihydroxyflavone, the dominant *nod*-gene inducer present as a conjugate in root extracts, showed that recently synthesized flavone molecules were present in both forms inside the root but only as the aglycone in the exudate (Table V). AOPP decreased specific activity of 4′,7-dihydroxyflavone in the conjugate fraction of the root extract by 89%, but it had no significant effect on the quantity of conjugate (data not shown) or specific activity of the aglycone form of that flavone in the extract (Table V). Specific activity of the flavone in the exudate from those same roots was decreased by 60%.

DISCUSSION

Release of the three major *nod*-gene-inducing flavonoids from roots of alfalfa seedlings is associated with concurrent biosynthesis. Each of the inducers was radiolabeled when roots of intact plants were treated with ^{14}C-phenylalanine, a flavonoid precursor (Table I). Furthermore, AOPP, which inhibits PAL activity (1), decreased incorporation of ^{14}C into exuded flavonoids (Table I) and reduced total release of those compounds (Table II). Similar results were obtained with exudates of excised plant organs (Tables III and IV).

Radiolabeling of the exuded *nod*-gene-inducing flavonoids in the presence of ^{14}C-phenylalanine indicates that their release is not tightly coupled to modification of preexisting

Table III. *Effects of the PAL Inhibitor AOPP on Incorporation of [U-^{14}C]-L-Phenylalanine into Flavonoids Released from Excised Organs of Alfalfa Seedlings*

Each value represents the mean of three replicates.

Treatment	Plant Organ	4′,7-Dihydroxy-flavanone	4,4′-Dihydroxy-2′-methoxychalcone	4′,7-Dihydroxy-flavone
			dpm·g fresh wt^{-1}	
Control	Root	3,680a[a]	2,160a	23,100a
Control	Hypocotyl	205,000b	6,900b	153,000b
Control	Cotyledon	34,000a	158a	87,600ab
AOPP	Root	149a	11a	1,150ab
AOPP	Hypocotyl	108a	92a	1,390b
AOPP	Cotyledon	9a	14a	234a
Treatment		51,300	885	31,500

[a] Values for a single flavonoid followed by the same letter did not differ significantly ($P < 0.05$) among organs within a treatment.

flavonoids. Phenylalanine enters the flavonoid biosynthetic pathway several steps before commitment to flavonoid biosynthesis (10). As a result, neither flavonoids that were present before treatment with ^{14}C nor alternative molecular structures which originated from them would be labeled. If preexisting flavonoids contributed to exudation, the exuded compounds also would lack label.

The factors regulating biosynthesis and release of the three major flavonoid *nod*-gene inducers probably differ. The total 4′,7-dihydroxyflavone exuded during three 8-h collections between 48 and 72 h (Fig. 1) was twice as great as that obtained with one 24-h collection during the same time period (20). In contrast, the quantities of 4′,7-dihydroxyflavanone and 4,4′-dihydroxy-2′-methoxychalcone were similar in both procedures (Fig. 1; 20). Also, $CuCl_2$ treatment of alfalfa seedlings greatly increased the magnitude of 4′,7-dihydroxyflavanone and 4′,7-dihydroxyflavone secretion by alfalfa roots but reduced exudation of 4,4′-dihydroxy-2′-methoxychalcone (Fig. 3). Wounding during preparation of plant parts may have had a similar effect with respect to the ratio of those compounds in exudates of plant parts (Table IV) compared with whole plants (Table II). Because the methoxychalcone would logically be formed by direct methoxylation of a chalcone, its biosynthesis should compete with phytoalexin synthesis, which proceeds through 4′,7-dihydroxyflavanone and is enhanced by both wounding (Tables II *versus* IV; 10) and $CuCl_2$ treatment (Fig. 3; 5, 10). The significance of enhanced flavone synthesis in the presence of $CuCl_2$ (Fig. 3) is unclear.

The release of flavonoids from alfalfa roots apparently is a specific process. If the process were associated with a general phenomenon, such as sloughing off of cells during root growth, then the flavonoids present in root extracts also should be found in exudates. That is not the situation. Large amounts of a formononetin conjugate (peak F_2, Fig. 2A) were observed in root extracts, but no formononetin or formononetin conjugate was found in exudates of normal roots (Figs. 1 and 3). In contrast, exudates of $CuCl_2$-treated roots did contain formononetin (Fig. 3), indicating the isoflavonoid could be released in substantial quantity. Additional evidence for specific release is the presence of *nod*-gene inducers in root extracts that are not found in root exudates (Fig. 2; 20). Formononetin did not induce *nod* genes.

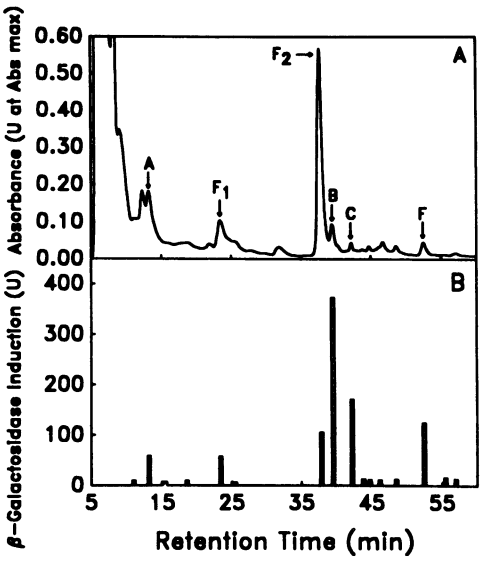

Figure 2. HPLC characteristics and *nod*-gene-induction assays of a flavonoid fraction from alfalfa root extract. A, A_{max} (230–400 nm) of extract fractionated on a reverse-phase C_{18} column in a methanol gradient. B, β-Galactosidase activity induced from *nodABC-lacZ* on pRmM57 in *R. meliloti* 1021 by fractions from chromatogram A. Regions indicated with letters contained the following compounds. A, conjugates of 4′,7-dihydroxyflavone and 4′,7-dihydroxyflavanone; B, 4,4′-dihydroxy-2′-methoxychalcone; C, 4′,7-dihydroxyflavone; F, formononetin; F_1 and F_2, unidentified conjugates of formononetin; F_1, 4′,7-dihydroxyflavanone.

Data from these experiments neither completely eliminate nor prove that flavonoid conjugates are involved in release of aglycones from alfalfa roots. Several points imply participation of conjugates: (i) Conjugates of both 4′,7-dihydroxyflavanone and 4′,7-dihydroxyflavone were detected in root extracts of 48-h-old alfalfa seedlings immediately prior to the time the corresponding aglycones were being exuded (Fig. 1;

Table IV. *Effects of the PAL Inhibitor AOPP on the Release of Flavonoids from Excised Organs of Alfalfa Seedlings*

Each value represents the mean of three replicates.

Treatment	Plant Organ	4′,7-Dihydroxy-flavanone	4,4′-Dihydroxy-2′-methoxychalcone	4′,7-Dihydroxy-flavone
			pmol·g fresh wt^{-1}	
Control	Root	737a[a]	26a	1,520a
Control	Hypocotyl	3,740c	29a	2,190a
Control	Cotyledon	1,570b	3a	2,380a
AOPP	Root	309a	0a	141a
AOPP	Hypocotyl	187a	20a	58a
AOPP	Cotyledon	104a	14a	25a
Treatment		265	25	437

[a] Values for a single flavonoid followed by the same letter did not differ significantly ($P < 0.05$) among organs within a treatment.

20). No conjugates of 4,4′-dihydroxy-2′-methoxychalcone were observed, but undetectable small quantities may have been present. (ii) At least one radioactive conjugate of 4′,7-dihydroxyflavone was isolated from extracts prepared from roots incubated with ^{14}C-phenylalanine (Table V). Probably only a small portion of the conjugate pool contributed aglycones for exudation, because the specific activity of 4′,7-dihydroxyflavone conjugate(s) extracted from alfalfa roots was significantly lower than that of the aglycone isolated from either extract or exudate (Table V). Because that pattern was accentuated greatly with AOPP treatment, a majority of recent flavonoid synthesis probably was exuded as aglycone rather than being converted to a conjugate. If the entire conjugate pool were closely involved in secretion of aglycones, then the specific activities of conjugated aglycones, internal aglycones, and exuded aglycones should have been similar.

Whether hypocotyls or cotyledons contribute to the synthesis of flavonoid *nod*-gene inducers that are released by roots of intact plants is unclear. Roots obviously can perform this function alone, because excised roots exuded radiolabeled forms of the normal *nod*-gene inducers when treated with ^{14}C-phenylalanine (Table III). However, cotyledons and hypocotyls also synthesized and released all three compounds (Tables III and IV). A conclusion is made more difficult by the technical problem of deciding the precise junction between the root and hypocotyl when those organs are excised. Nevertheless, the relative ratio of the radioactive *nod*-gene inducers secreted from roots of whole plants was closer to that of excised roots than for hypocotyls or cotyledons (Tables I and III).

Results from this study suggest the following preliminary model. Flavonoid *nod*-gene inducers are synthesized as aglycones in the cytoplasm of root cells. The aglycones can then be exuded into the rhizosphere through a specific process or stored in vacuoles as conjugates. The conjugated flavonoids can be released later through the actions of specific hydrolases.

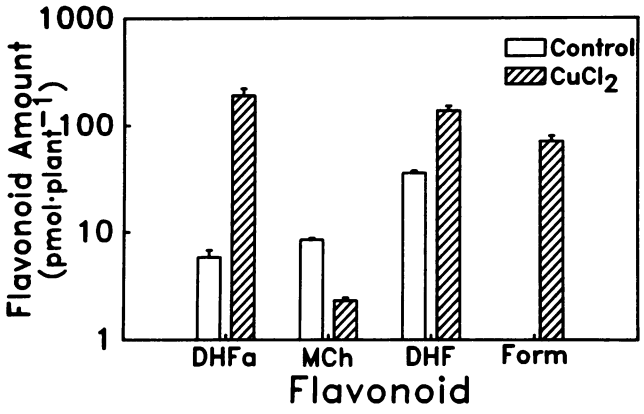

Figure 3. Effects of $CuCl_2$ on the release of flavonoid *nod*-gene inducers and formononetin from roots of intact alfalfa seedlings. Root exudates produced 16 to 24 h after treating with $CuCl_2$ contained the indicated amounts of DHFa (4′,7-dihydroxyflavanone), MCh (4,4′-dihydroxy-2′-methoxychalcone), DHF (4′,7-dihydroxyflavone), and Form (formononetin). No formononetin was detected in root exudates of the control seedlings.

Table V. *Effects of the PAL Inhibitor AOPP on Specific Activity of 4′,7-Dihydroxyflavone Exuded as the Aglycone from Roots of Intact Alfalfa Seedlings or Extracted from Roots as the Aglycone or Conjugated Form after Incubation with [U-^{14}C]-L-Phenylalanine*

No 4′,7-dihydroxyflavone conjugate was detected in the root exudate. Conjugates, presumably glycosides, were acid hydrolyzed to 4′,7-dihydroxyflavone prior to quantification of amounts and associated radioactivity. Each value represents the mean of three samples.

Treatment	Extract		Exudate Aglycone	Fraction $LSD_{(0.05)}$
	Conjugate	Aglycone		
	dpm·pmol^{-1}			
Control	5.6	9.2	11.7	2.6
AOPP	0.6	7.0	4.7	2.4
$LSD_{(0.05)}$	1.5	NS	1.1	

Such a model predicts that factors which affect flavonoid biosynthesis should also directly impact release of *nod*-gene inducers into the rhizosphere. Although large quantities of *nod*-gene-inducing flavonoids are released from germinating alfalfa seeds (9), the synthesis and exudation processes measured in the 72-h-old seedlings of this study may be important for nodule formation. The *nod*-gene-inducing flavonoids studied here are also present in root exudates of mature alfalfa plants (UA Hartwig, unpublished results), and thus nodulation of older roots far removed from the original seed zone probably depends on processes very similar to those described here.

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