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Chrysoeriol and Luteolin Released from Alfalfa Seeds Induce *nod* Genes in *Rhizobium meliloti*[1]

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ABSTRACT

Flavonoid signals from alfalfa (*Medicago sativa* L.) seed and root exudates induce transcription of nodulation (*nod*) genes in *Rhizobium meliloti*. The flavone luteolin previously was isolated from alfalfa seeds by other workers and identified as the first *nod* gene inducer for *R. meliloti*. Our recent study of 'Moapa 69' alfalfa root exudates found no luteolin but did identify three other *nod* gene inducers: 4,4′-dihydroxy-2′-methoxychalcone, 4′,7-dihydroxyflavone, and 4′,7-dihydroxyflavanone. The goal of the current study was to identify and quantify *nod* gene-inducing flavonoids that may influence *Rhizobium* populations around a germinating alfalfa seed. Aqueous rinses of Moapa 69 alfalfa seeds were collected and assayed for induction of a *nodABC-lacZ* fusion in *R. meliloti*. During the first 4 hours of imbibition, total *nod* gene-inducing activity was released from seeds at 100-fold higher rates than from roots of 72-hour-old seedlings. Five flavonoids were purified and identified by spectroscopic analyses (ultraviolet/visible absorbance, proton nuclear magnetic resonance, and mass spectroscopy) and comparison with authentic standards. Two very active *nod* gene-inducing flavonoids, chrysoeriol (3′-methoxyluteolin) and luteolin, were identified in seed rinses. Luteolin required a higher concentration (18 nanomolar) than chrysoeriol (5 nanomolar) for half-maximum induction of *nodABC-lacZ* in *R. meliloti*, and both were less active than 4,4′-dihydroxy-2′-methoxychalcone (2 nanomolar) from root exudates. Seeds exuded three other luteolin derivatives: luteolin-7-*O*-glucoside, 5-methoxyluteolin, and 3′,5-dimethoxyluteolin. Their combined quantities were 24-fold greater than that of luteolin plus chrysoeriol. Most *nod* gene-inducing activity of these luteolin derivatives apparently is associated with degradation to luteolin and chrysoeriol. However, their presence in large quantities suggests that they may contribute significantly to *nod* gene-inducing activity in the soil. These results indicate the importance of germinating seeds as a source of *nod* gene-inducing flavonoids and emphasize the quantitative and qualitative differences in those compounds around the seed and root.

Establishment of the N_2-fixing symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa* L.) requires a number of interactions between the two organisms. After the plant releases a signal that induces transcription of the bacterial nodulation genes *nodABC* (22), rhizobial products induce root hair curling (17) and cortical cell divisions (8). The first molecule showing *nod* gene-inducing activity in *R. meliloti* was isolated from alfalfa seeds and identified as 3′,4′,5,7-tetrahydroxyflavone, a compound known as luteolin (23). Recent studies of root exudates from 72-h-old 'Moapa 69' alfalfa seedlings identified 4,4′-dihydroxy-2′-methoxychalcone, 4′,7-dihydroxyflavone, and 4′,7-dihydroxyflavanone as active *nod* gene inducers, but no luteolin was detected (20). Numerous flavonoids from various legumes have been reported as active *nod* gene inducers (4, 9, 18, 25, 26, 29), but qualitative and quantitative differences between compounds actually released from seeds and roots have not been described.

The presence of particular flavonoids inside plants cannot be taken as evidence of their release into exudates. Yelton *et al.* (28) observed that extracts, but not exudates, of some plant species induced *nod* genes in *R. meliloti*. Presumably, host plant factors controlling the synthesis and release of flavonoids can affect how rhizobia and legumes interact to form root nodules. Thus, the long list of flavonoids extracted from '*Medicago* × *varia* Martyn' seeds (10) (apparently a hybrid of *M. sativa* L. × *M. falcata* L. [14]) does not indicate which compounds will be available to rhizobial cells near a seed in the soil. To understand how alfalfa and *R. meliloti* interact, a more complete identification of *nod* gene-inducing flavonoids released from alfalfa seeds is required. The present study was designed to identify *nod* gene-inducing flavonoids released from Moapa 69 alfalfa seeds and to quantify their importance relative to other *nod* gene inducers released from roots (20).

MATERIALS AND METHODS

Preparation of Exudates

Alfalfa (*Medicago sativa* L., cv 'Moapa 69') seeds (1.0 g, about 400 seeds) were scarified, surface-sterilized for 3 min in 70% ethanol, washed three times with sterile water, and imbibed in aerated, sterile water. After 4 h, seeds were removed, and the remaining solution, termed seed exudate, was diluted 1:1 with methanol for quantitative HPLC measurements and assays or dried under vacuum for large-scale purification studies. Root exudate, described previously (20), consisted of an aerated, hydroponic solution that had bathed roots of seedlings developing from the seeds after rinsing for 24 h. The hydroponic containers, over which seedlings were suspended (20), were kept at 25°C in a laminar-flow hood with irradiance of 60 $\mu E \cdot m^{-2} \cdot s^{-1}$ and a 12-h photoperiod.

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Biological Activities

The *nod* gene-inducing activity of compounds was assayed as β-galactosidase activity transcribed from the *nodABC-lacZ* fusion on plasmid pRmM57 in *R. meliloti* strain 1021 (22). Assays (22) were done under conditions described previously (20), and activity was recorded in Miller units (21) after subtracting uninduced control values. The assay strain, *R. meliloti* 1021pRmM57, was generously provided by Dr. S. R. Long (Stanford University).

Concentrations of all flavonoid compounds used in assays were determined spectrophotometrically with the following extinction coefficients (log ε): luteolin, 4.17 at 350 nm in 95% ethanol (19); 4,4′-dihydroxy-2′-methoxychalcone, 4.25 at 349 nm in methanol (6); 3′,5-dimethoxyluteolin, 4.18 at 337 nm in methanol (1). Log ε values for chrysoeriol, 4.35 at 347 nm in methanol, 5-methoxyluteolin, 4.32 at 341 nm in methanol, and luteolin-7-*O*-glucoside, 4.37 at 348 nm in 95% ethanol, were determined gravimetrically on a Cahn 28 Automatic Electrobalance (Cahn Instruments Inc., Cerritos, CA). Aliquots of standardized flavonoid stock solutions were dried under vacuum and stored at −20°C. Before each assay, flavonoid compounds were dissolved in methanol and then diluted into a pH 7.0 phosphate buffer. Methanol was removed under vacuum before bacteria were added to the assay mixtures.

Purification of *nod* Gene Inducers

Small-scale quantification studies were done with 100-μL aliquots of fresh seed exudate that were adjusted 1:1 with methanol and injected into a Waters HPLC system (Millipore Corp., Milford, MA) equipped with a 250 × 4.6 mm Lichrosorb 5RP18 analytical reverse-phase column (Phenomenex, Rancho Palos Verdes, CA). The column was eluted at 0.5 $mL \cdot min^{-1}$ from 0 to 70 min with a linear gradient from 30:67.5:2.5 (v/v/v) methanol:water:acetic acid to 60:37.5:2.5 (v/v/v) methanol:water:acetic acid. From 70 to 90 min the analysis continued isocratically with 60:37.5:2.5 (v/v/v) methanol:water:acetic acid. Flavonoids were monitored with a Waters 990 photodiode array detector which charted the maximum absorbance between 230 and 400 nm every second with a resolution of 1.4 nm. Eluant fractions were collected every 30 s and combined when associated with visible peaks. Those fractions were dried under vacuum, and portions (*e.g.* 20% of a peak) were dissolved in 100 μL of 0.1 M sodium phosphate buffer [pH 7.0] for *nod* gene induction assays. As each *nod* gene-inducing flavonoid was identified, the quantity present in seed exudate was determined relative to known amounts of authentic standards by applying the integrator function within the Waters 990 software package and appropriate extinction coefficients.

Large-scale purification of active *nod* gene inducers was initiated by redissolving dried seed exudate in 50% methanol and partitioning against equal volumes of hexane to remove lipids. The methanolic phase was centrifuged, and 500-μL aliquots were separated on a 250 × 10 mm Lichrosorb 5RP18 semipreparative reverse-phase column (Alltech Associates, Inc.) with the above methanol gradient and a flow rate of 2 $mL \cdot min^{-1}$. Fractions of interest were dried under N_2 at 45°C, redissolved in 50% methanol, and rechromatographed isocratically in 60:37.5:2.5 (v/v/v) methanol:water:acetic acid on the same column.

Identification of *nod* Gene Inducers

UV/visible spectral shift analyses were conducted with a Lambda 6 dual beam spectrophotometer (Perkin Elmer, Norwalk, CT) and interpreted by established methods (19).

COSY[2] and one-dimensional proton-NMR experiments were done in [U-^{2}H]methanol on a Nicolet NT-360 spectrometer at the NMR facility, University of California at Davis. Spectra were referenced to the methyl peak (3.30 ppm) of methanol. A 60°-pulse of 3.6 to 4.3 μs, with a time delay of 1 s and a sweep width of ±5000 Hz was used.

Electron ionization MS data were collected by the staff of the Facility for Advanced Instrumentation, University of California, Davis, using a solids probe to introduce the samples into a Trio-2 MS (VG Masslab, Altrincham, UK).

The sugar determination was generously made by Dr. J. M. Labavitch (UC Davis) after acid hydrolysis with TFA (3).

Tentative identifications of all compounds were confirmed by comparison with authentic standards using HPLC cochromatography, UV/visible absorbance shift tests, single-dimension proton-NMR spectroscopy, and tests for biological activity in the *nodABC-lacZ* induction assay. Authentic standards were obtained from the following sources: luteolin, luteolin-7-*O*-glucoside, and 4′,7-dihydroxyflavone (Spectrum Mfg. Corp., Gardena, CA); chrysoeriol (3′-methoxyluteolin) (Apin Chemicals Ltd., Oxon, UK); apigenin (Aldrich Chemical Co., Milwaukee, WI). Prof. Dr. H. Geiger, Universität Hohenheim, Stuttgart, FRG, generously donated samples of 5-methoxyluteolin and 3′,5-dimethoxyluteolin. When necessary, standards were purified by HPLC.

RESULTS

Release of *nod* Gene-Inducing Activity

During the first 4 h of imbibition, Moapa 69 alfalfa seeds released *nod* gene-inducing activity at a 100-fold greater rate than roots of 72-h-old seedlings (Fig. 1). When exudates from a 0 to 4 h seed rinse were fractionated on the HPLC (Fig. 2A) and 20% of each peak was assayed for *nod* gene induction, the amount of inducing activity (Fig. 2B) relative to the quantity of compound present in the exudate (Fig. 2A) was much higher for peaks 4 and 5 than for 1, 2, and 3.

Compound Identifications

Compound 4

UV/visible spectral shift experiments showed that CH_3ONa produced a strong and stable bathochromic shift of band I (300–380 nm) with no decrease in intensity (Table I), which

[2] Abbreviations: COSY, two-dimensional homonuclear shift correlation spectroscopy; δ_H, chemical shift of proton [ppm]; 1H, one proton, 2H, two protons, etc.; C-5, carbon number five, etc.; *d*, doublet; *dd*, double doublet; *s*, singlet; *m*, multiplet; J, coupling constant; *m/z*, mass charge ratio.

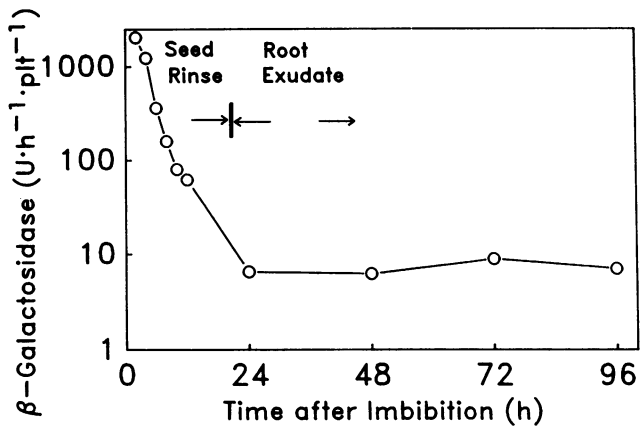


Figure 1. Release of *nod* gene inducing activity from alfalfa seeds (0–24 h) and roots (24–96 h). Seed and root exudates were assayed for their capacity to induce transcription of β-galactosidase from *nodABC-lacZ* in *R. meliloti* 1021pRmM57. Values represent mean ± SE of release rates per plant (plt) of four replicates. SE bars are obscured by symbols.

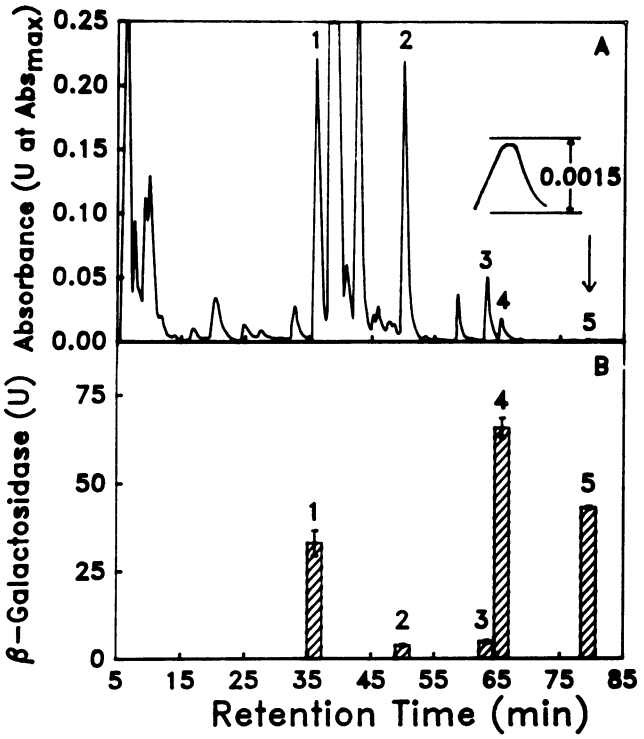


Figure 2. HPLC characteristics and *nod*-induction assays of alfalfa seed exudate released during the first 4 h of imbibition. A, A_{max} (260–400 nm) of exudates fractionated on a reverse-phase C_{18} column. The inset at 0.0015 AU full scale represents peak 5. B, β-Galactosidase activity induced from *nodABC-lacZ* on pRmM57 in *R. meliloti* 1021 by fractions from chromatogram A. Values represent the mean ± SE of three replicates. Peaks were subsequently identified as: 1, luteolin-7-*O*-glucoside; 2, 5-methoxyluteolin; 3, 3′,5-dimethoxyluteolin; 4, luteolin; and 5, chrysoeriol (3′-methoxyluteolin).

is characteristic of a 4′-hydroxyl (C numbers indicated on Fig. 3). The bathochromic shift of band II (240–280 nm) in the presence of CH_3COONa suggested the presence of a 7-hydroxyl. The bathochromic shift of band II caused by $AlCl_3$/HCl indicated a 5-hydroxyl, and the bathochromic shift of band I with CH_3COONa/H_3BO_3 supported the presence of an *o*-dihydroxy configuration on the B-ring (C-2′ to C-6′). One-dimensional proton-NMR experiments identified the following proton resonances: δ_H ppm ([U-^{2}H]methanol): 7.34 to 7.32 (2H, *m*, C-6′, C-2′); 6.84 (1H, *d*, J = 9.9 Hz, C-5′); 6.41 (1H, *s*, C-3); 6.26 (1H, *d*, J = 2.9 Hz, C-8); 6.06 (1H, *d*, J = 2.9 Hz, C-6). A cross-peak between C-6′, C-2′, and C-5′ on the COSY indicated that both C-6′ and C-5′ were unsubstituted (data not shown). MS data showed mol wt = 286 with the following major fragments: *m/z* 258, 153, and 134. All data supported a tentative identification of compound 4 as luteolin (3′,4′,5,7-tetrahydroxyflavone).

Compound 5

UV/visible spectral shift data of this compound were similar to those measured for compound 4, except that the lack of a bathochromic shift of band I in the presence of NaOAc/H_3BO_3 suggested the absence of an ortho-dihydroxy configuration (Table I). One-dimensional proton-NMR experiments identified the following proton resonances: δ_H ppm ([U-^{2}H]methanol): 7.50 (2H, *dd*, J = 8.8, 1.5 Hz, C-6′, C-2′); 6.93 (1H, *d*, J = 8.4 Hz, C-5′); 6.63 (1H, *s*, C-3); 6.46 (1H, *d*, J = 2.0 Hz, C-8); 6.20 (1H, *d*, J = 2.1 Hz, C-6); 3.96 (3H, *s*, -OCH_3). Data from UV/visible spectral experiments suggested the methyl peak (3.96 ppm) was at the C-3′ position. That possibility was supported by COSY data which showed an unsubstituted C-5′, C-6′ configuration (data not shown). MS data indicated mol wt = 300 with the following major fragments: *m/z*, 286, 152, 147, 137, and 133. Apparent transmethylation reactions (11) gave a significant peak at *m/z* 314. All data supported a tentative identification of compound 5 as chrysoeriol (3′-methoxyluteolin).

Compound 1

The UV/visible spectral shift data from this compound were consistent with those from luteolin, except that the minor bathochromic shift of band II in CH_3COONa suggested the lack of a C-7 hydroxyl (Table I). One-dimensional proton-NMR experiments identified the following proton resonances: δ_H ppm ([U-^{2}H]methanol): 7.41 (2H, *dd*, J = 8.1, 7.5 Hz, C-6′, C-2′); 6.90 (1H, *d*, J = 7.9 Hz, C-5′); 6.80 (1H, *d*, J = 1.8 Hz, C-8); 6.60 (1H, *s*, C-3), 6.50, (1H, *d*, J = 1.7 Hz, C-6); 3.94–3.69 (6H, *m*, glucosyl). A multiplet between 3.94 and 3.69 ppm suggested the presence of a sugar, and gas chromatographic analyses after hydrolization with TFA showed the presence of glucose. These data supported a tentative identification of compound 1 as luteolin-7-*O*-glucoside.

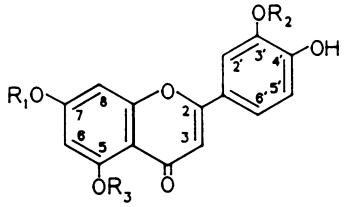
Compound 2

UV/visible spectral shift data of this compound were consistent with those from luteolin (compound 4) except the lack of a bathochromic shift in band II with $AlCl_3$/HCl relative to

Table I. *UV/Visible Absorption Maxima of Purified Nod Gene Inducers from Figure 2*
Compounds were treated with standard shift reagents (19) in methanol.

Shift Reagent	Compound				
	1 Luteolin-7-*O*-glucoside	2 5-Methoxyluteolin	3 3′,5-Dimethoxyluteolin	4 Luteolin	5 Chrysoeriol
			γ max (nm)		
Methanol	255,265,349	244,262,341	240,264,337	254,266,293sh[a],349	249sh,268,347
CH_3ONa	267,401	262,313sh,389	260,270sh,317sh,392	267,331sh,402	264,275sh,326sh,406
$AlCl_3$	273,295sh,332,428	265,301sh,368,460	241,264,337,413	273,301sh,331,426	262,297,362sh,389
$AlCl_3$/HCl	271,294sh,361,388	263,300sh,344,416	265,301,342,415	275,295sh,358,385	276,296,354,390
CH_3COONa	263,369sh,407	267,316sh,383	260,268,312,386	269,327sh,398	266,275sh,325,402
CH_3COONa/H_3BO_3	260,373	253,359	264,339	261,372,431	269,350

[a] sh, spectral shoulder.



$R_1 = R_2 = R_3 = H$: Luteolin

R_1 = Glucose; $R_2 = R_3 = H$: Luteolin-7-O-glucoside

$R_1 = H$; $R_2 = R_3 = CH_3$: 3′,5-Dimethoxyluteolin

$R_1 = R_3 = H$; $R_2 = CH_3$: 3′-Methoxyluteolin

$R_1 = R_2 = H$; $R_3 = CH_3$: 5-Methoxyluteolin

Figure 3. Structures of five flavonoids identified in alfalfa seed exudate. Structures correspond to compounds in Figure 2 as follows: 1, luteolin-7–*O*-glucoside; 2, 5-methoxyluteolin; 3, 3′,5-dimethoxyluteolin; 4, luteolin; and 5, chrysoeriol (3′-methoxyluteolin).

methanol suggested the C-5 position existed in either an unsubstituted or a methoxylated condition (Table I). One-dimensional proton-NMR experiments identified the following proton resonances: δ_H ppm ([U-^{2}H]methanol): 7.34 (2H, *dd*, J = 2.0, 2.9 Hz, C-6′, C-2′); 6.88 (1H, *d*, J = 9.1 Hz, C-5′); 6.54 (1H, *s*, C-8); 6.48 (1H, *s*, C-3); 6.41 (1H, *d*, J = 1.7 Hz, C-6); 3.87 (3H, *s*, -OCH_3). COSY results indicated that C-5′, C-6′ were unsubstituted (data not shown). The position of the methoxy group was established by the absence of a proton resonance associated with C-5 and by a strong, bright-blue florescence under 360 nm irradiance with and without ammonia (26). The MS data showed a mol wt = 300 with the following major fragments: *m/z* 286, 285, 167, 137, and 133. Apparent transmethylation reactions (11) gave a significant peak at *m/z*, 314. These data supported a tentative identification of compound 2 as 5-methoxyluteolin.

Compound 3

UV/visible spectral shift data were consistent with those from 5-methoxyluteolin (compound 2) except the lack of a bathochromic shift of band I in CH_3COONa/H_3BO_3 suggested the absence of an *o*-dihydroxy configuration on the B-ring (Table I). This compound also showed a bright blue fluorescence under 360 nm irradiation. One-dimensional proton-NMR experiments identified the following proton resonances: δ_H ppm ([U-^{2}H]methanol): 7.47 (2H, *dd*, J = 2.1, 2.2 Hz, C-6′, C-2′); 6.92 (1H, *d*, J = 8.5 Hz, C-5′); 6.57 (2H, *d*, J=2.7 Hz, C-8, C-3); 6.42 (1H, *d*, J = 2.1 Hz, C-6); 3.92 (6H, *d*, J = 24.1 Hz, OCH_3-, -OCH_3). Because the COSY data indicated C-5′ and C-6′ were unsubstituted, the presence of two methoxy peaks in the single-dimension NMR experiment was consistent with C-3′ and C-5 methoxylation. MS data showed mol wt = 314 with the following major fragments: *m/z* 299, 285, 167, 148, and 133. Apparent transmethylation reactions (11) gave a significant peak at *m/z* 328. These data supported a tentative identification of compound 3 as 3′,5-dimethoxyluteolin.

Other Compounds

When high concentrations of seed rinses were examined on the semipreparative HPLC column, two very small peaks with retention times of 62 and 78 min were tentatively identified as 4′,7-dihydroxyflavone and apigenin, respectively, by UV/visible spectra and cochromatography with authentic standards (data not shown).

Verification of Identifications

Chemical data for compounds 1 to 5 closely matched those reported in the literature for the same molecules (1, 16, 19, 27). When compounds 1 to 5 were compared with authentic standards, identical retention times in the HPLC gradient, equivalent UV/visible spectral shift data, and comparable one-dimensional NMR results confirmed the identifications.

Comparisons between induction of *nodABC-lacZ* by authentic and putative samples of compounds 1 to 5 showed similar concentration dependencies (Table II) when both were purified on the standard HPLC methanol gradient immediately before assaying. Tests with authentic samples of luteolin and chrysoeriol (Fig. 4) showed that both are less active *nod* gene inducers than 4,4′-dihydroxy-2′-methoxychalcone, which was identified in alfalfa seedling root exudates (20). Chrysoeriol gave reproducibly stronger induction than luteolin. The concentrations required for half-maximum activities

Table II. *Comparison of Nod Gene Inducing Activity for Putative and Authentic Flavonoids*
Values represent means for three replicates. Compound numbers refer to Figure 2.

Concentration	Compound									
	1 Luteolin-7-*O*-glucoside		2 5-Methoxyluteolin		3 3′,5-Dimethoxyluteolin		4 Luteolin		5 Chrysoeriol	
	Putative	Authentic	Putative	Authentic	Putative	Authentic	Putative	Authentic	Putative	Authentic
nM	*units β-galactosidase*									
0.84									6.1	6.7
2.1									15.6	21.1
4.2							4.9	4.9	31.9	33.9
8.4							12.2	11.2	45.7	50.5
21							29.2	29.6	63.6	61.5
42							55.1	53.6		
84	1.4	2.0	1.1	0.6	2.7	2.3	66.2	69.3		
210	5.1	5.2	1.6	1.1	3.4	3.2				
840	28.8	29.5	3.0	2.3	5.0	8.6				
2,100	53.6	58.2	5.6	4.6	9.7	12.6				
LSD (0.01)	4.8		0.9		2.9		3.9		5.9	

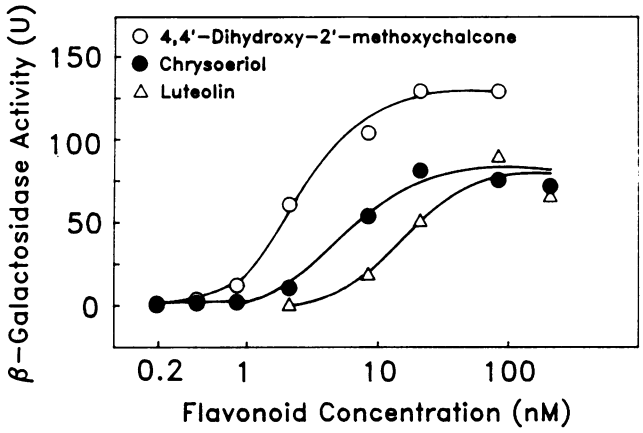


Figure 4. Induction of *R. meliloti nod* genes by flavonoids in alfalfa seed exudate. Compounds were tested for their capacity to induce transcription of β-galactosidase from *nodABC-lacZ* on pRmM57 in *R. meliloti* 1021. Values represent the mean ± SE of three replicates. SE bars are obscured by symbols. The 4,4′-dihydroxy-2′-methoxychalcone was not detected in seed exudate but was included as a control representing the strongest *nod* gene inducer from root exudate (20).

were 2, 5, and 18 nM for 4,4′-dihydroxy-2′-methoxychalcone, chrysoeriol, and luteolin, respectively.

Direct tests indicated that the *nod* gene-inducing activity associated with high concentrations of luteolin-7-*O*-glucoside, 5-methoxyluteolin, and 3′,5-dimethoxyluteolin resulted mostly from artifactual degradation and/or colorimetric interference with the quantification of β-galactosidase activity. When authentic samples of the compounds were subjected to variations of the normal purification methods (freeze-dried instead of vacuum-dried with or without acetic acid in the HPLC solvent) and then assayed for *nodABC-lacZ* induction, the concentration dependencies for *nod* gene induction closely matched those in Table II (data not shown). However, when authentic luteolin-7-*O*-glucoside samples from those treatments were rechromatographed isocratically in 60:37.5:2.5 methanol:water:acetic acid, a detectable peak with the retention time and UV/visible absorbance spectrum of luteolin was present in all cases. Integration of the peaks for the authentic compound and the luteolin-like breakdown product indicated that approximately 4% of the luteolin-7-*O*-glucoside had been degraded. Comparable chromatographic measurements of 5-methoxyluteolin and 3′,5-dimethoxyluteolin showed no detectable luteolin or chrysoeriol products.

Colorimetric interference in the *nod* gene induction assay at 2100 nM concentration (Table II) was indicated by incubation of luteolin-7-*O*-glucoside, 5-methoxyluteolin, and 3′,5-dimethoxyluteolin in the normal assay without bacteria. Under those conditions, 2100 nM concentrations of the three compounds produced increases in 420-nm absorbance equivalent to 9.1 ± 0.5, 5.5 ± 0.8, and 6.0 ± 0.3 U of β-galactosidase activity (mean ± SE), respectively.

Amounts of Compounds

The 4-h aqueous rinse of Moapa 69 seeds analyzed in Figure 2 contained the following amounts of *nod* gene-inducing flavonoids (pmol·seed^{-1}): luteolin-7-0-glucoside, 3170; 5-methoxyluteolin, 3300; 3′,5-methoxyluteolin, 1190; luteolin, 290; chrysoeriol, 38; apigenin, <3; and 4′,7-dihydroxyflavone, <2. The trace amounts of the latter two compounds were estimated by relating their peak areas to that of chrysoeriol and correcting for differences in extinction coefficients.

DISCUSSION

Results from this study indicate that alfalfa seeds release the strong *nod*-gene-inducing compound chrysoeriol (3′-methoxyluteolin), in addition to the previously reported luteolin (23). Chrysoeriol produces a half-maximum induction of the *nodABC-lacZ* fusion in *Rhizobium meliloti* 1021pRmM57 at about one-third the concentration required for luteolin (Fig. 4). Although chrysoeriol was identified before

in *Medicago* seed extracts (10), no biological role for this molecule has been reported in plants.

The importance of luteolin in the *Rhizobium*-alfalfa system, which was suggested earlier (23), is supported and extended by the demonstration that alfalfa seeds release large quantities of three other luteolin derivatives: luteolin-7–*O*-glucoside (compound 1), 5-methoxyluteolin (compound 2), and 3′,5-dimethoxyluteolin (compound 3) (Figs. 2 and 3). These compounds showed apparent *nod* gene-inducing activity at high concentrations (*e.g.* 840–2100 nM, Table II), but careful tests indicated that those results were largely associated with degradation (luteolin-7–*O*-glucoside) and colorimetric interference with the *nod*-induction assay at 2100 nM (all three compounds). The possibility that rhizobial enzymes produced luteolin and/or chrysoeriol during the assay was not completely eliminated, but we conclude that luteolin-7–*O*-glucoside, 5-methoxyluteolin, and 3′,5-dimethoxyluteolin are essentially inactive as inducers of *nod* genes in *R. meliloti* 1021pRmM57. The release of luteolin from luteolin-7-*O*-glucoside observed here indicates that a previous report (24) in which samples of flavonoid glucosides were purified and examined on HPLC before, but not after, assaying must be reexamined.

Trace amounts of two other flavonoids, apigenin and 4′,7-dihydroxyflavone, which were previously reported in *Medicago* seed extracts (10), also were tentatively identified here in Moapa 69 seed exudates. These compounds are very weak inducers of *nodABC-lacZ* in *R. meliloti* (20, 23). Apparently, the minute amounts in the original seed exudate sample (Fig. 2) prevented detection of any *nod*-inducing activity during the initial stages of this study. The presence of 4′,7-dihydroxyflavone, but not apigenin, in Moapa 69 seedling root exudates (20) distinguishes it as the only *nod* gene inducer found thus far in both alfalfa seed and root exudates.

Data from this study expressed on a per-plant basis indicate that *nod* gene inducers are released during the earliest stages of seed imbibition at rates much higher than those measured for young seedling roots (Fig. 1). This rapid rate of release, which was measured even during the first hour of imbibition, suggests that significant quantities of the compounds may be solubilized from preexisting storage pools without enzymatic conversion. This concept is consistent with the fact that all *nod* gene inducers identified from alfalfa seed exudate in this study also were extracted from *Medicago* seeds (10). The rapid release of nod gene inducers from seeds (Fig. 1) does not diminish the importance of similar compounds exuded at lower rates from roots of the same cultivar (20). The rate at which *nod* gene-inducing flavonoids are exuded from seeds decreases within a few hours, while seedling roots release *nod* gene inducers over a period of several days. Calculations from both studies suggest that during the first 8 d of growth, Moapa 69 roots exude about 25% as much total *nod* gene-inducing activity as seeds do during the first 24 h.

Structural differences between the major *nod* gene inducers released by alfalfa seeds, which have substitutents at the 3′, 4′, 5, and 7 positions (Fig. 3), and those exuded by roots, which have 4′,7-substitution patterns (20), may be important for understanding how rhizosphere chemistry influences *R. meliloti* and symbiotic N_2 fixation in alfalfa. For example, combinations of luteolin, the dominant *nod* gene inducer from seeds, and 4,4′-dihydroxy-2′-methoxychalcone, the strong *nod* gene inducer from alfalfa root exudates, produced a synergistic increase in transcription of the *nodABC-lacZ* fusion in *R. meliloti* 1021pRmM57 (13). Since it is known that *R. meliloti* has three functional copies of *nodD* (12, 15), that effect may operate through the capacity of these molecules to interact with different *nodD* genes (UA Hartwig, unpublished data). This hypothesis suggests that a region of extremely strong *nod* gene-inducing activity exists where flavonoids from seed and root exudation meet and may partially explain the presence of numerous root nodules near the top of the alfalfa primary root.

The presence of luteolin derivatives in the seed exudate also may have significant effects on how alfalfa interacts with *R. meliloti* in the soil. Results showing that 1 nM luteolin is a chemoattractant for *R. meliloti* (5) suggest that the luteolin-7–0-glucoside released from alfalfa seeds may be a particularly important compound. The relatively greater solubility of that compound in aqueous solution, compared with luteolin, indicates that under appropriate conditions in the rhizosphere it could move greater distances than luteolin. In the presence of microbial glycosidases (2), the luteolin aglycone would be released. Thus, a concentration gradient of luteolin extending away from the plant could stimulate rhizobia to move toward the plant where transcription of *nod* genes could initiate the symbiosis. Methoxylated derivatives of luteolin may also be converted to luteolin or chrysoeriol (from 3′,5-dimethoxyluteolin) by microbial demethylases (7) to produce active *nod* gene inducers. The potential importance of 3′,5-dimethoxyluteolin in the soil remains to be proven, but the known instability of the 5-methoxyl relative to the 3′-methoxyl group (11) suggests that this compound may serve as a significant source of chrysoeriol.

The primary significance of this report is that the present data complement information on *nod* gene inducers from alfalfa seedling roots (20) to offer the first extensive data base for understanding how seeds and roots of one legume send a complex set of chemical signals to potential *R. meliloti* symbionts. It seems doubtful that the diversity of signals has been completely described. There is, for example, no information available yet on the structure of *nod* gene-inducing flavonoids released from roots produced later in the life of the perennial alfalfa plant. Identifying those molecules, describing the conditions controlling the release of all *nod* gene inducers, and defining the molecular effects of *nod* gene inducers on *R. meliloti* offer intriguing opportunities for rhizosphere biologists.

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