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Alfalfa (*Medicago sativa* L.) Root Exudates Contain Isoflavonoids in the Presence of *Rhizobium meliloti*[1]

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Root exudates of alfalfa (*Medicago sativa* L.) inoculated with symbiotic *Rhizobium meliloti* bacteria contained three isoflavonoids that were not found in exudates of uninoculated plants. Data from proton nuclear magnetic resonance, mass spectrometry, and ultraviolet-visible absorbance analyses indicated that root exudates of inoculated plants contained aglycone and glycoside forms of the phytoalexin medicarpin and a formononetin-7-*O*-(6″-*O*-malonylglycoside), a conjugated form of the medicarpin precursor formononetin. The medicarpin molecules did not induce *nod* gene transcription in *R. meliloti*, but the formononetin-7-*O*-(6″-*O*-malonylglycoside) induced *nod* genes regulated by both NodD1 and NodD2 proteins in *R. meliloti*. Hydrolysis of either the malonyl or the glycosyl linkage from the formononetin conjugate eliminated *nod* gene-inducing activity. The *nod* gene-inducing activity of crude root exudates was increased 200 and 65% upon inoculation with *R. meliloti* or *R. leguminosarum* bv *phaseoli*, respectively. When root exudate from uninoculated alfalfa was incubated with *R. meliloti*, high performance liquid chromatography analyses showed no evidence that bacterial metabolism produced medicarpin. These results indicate that alfalfa responds to symbiotic *R. meliloti* by exuding a phytoalexin normally elicited by pathogens and that the microsymbiont can use a precursor of the phytoalexin as a signal for inducing symbiotic *nod* genes.

Alfalfa (*Medicago sativa* L.) grown in the absence of its N_2-fixing symbiont *Rhizobium meliloti* releases five flavonoids (Peters et al., 1986; Maxwell et al., 1989; Hartwig et al., 1990a) and two betaines (Phillips et al., 1992) that induce transcription of nodulation (*nod*) genes in that bacterium. Studies with white clover (Rolfe et al., 1988) and *Vicia sativa* subsp. *nigra* (van Brussel et al., 1990) showed that inoculating with an infective rhizobial symbiont increased *nod* gene-inducing activity associated with the root by as much as 10-fold. Analyses of the *Vicia* root exudates identified eight *nod* gene-inducing flavonoids that increased in the presence of *Rhizobium*, but there was no evidence that *Vicia* inoculated with *R. leguminosarum* bv *viciae* released other new compounds that failed to induce *nod* genes (Recourt et al., 1991). Whether alfalfa roots release additional *nod* gene-inducers in the presence of *R. meliloti* is unknown.

Microbes often elicit phytoalexins in legumes (Dixon, 1986), but the role of these compounds in development of N_2-fixing root nodules is unclear. Soybeans (*Glycine max* [L.] Merr.) infected with *Bradyrhizobium japonicum* produce the isoflavonoid phytoalexin glyceollin (Parniske et al., 1990), and *B. japonicum* cells develop resistance to that compound when exposed to its biosynthetic precursors (Parniske et al., 1991; Kape et al., 1992). The fact that there is no evidence that *Vicia* roots treated with *Rhizobium* exuded isoflavonoid phytoalexins (Recourt et al., 1991) is intriguing because many of the *Rhizobium*-dependent compounds that appeared were 5-deoxy flavonoids that could be metabolized to medicarpin, a common phytoalexin in *V. faba* (Hargreaves et al., 1976). Alfalfa cells treated with elicitors from a pathogen produce medicarpin (Kessmann et al., 1990), but whether *R. meliloti* induces alfalfa to exude medicarpin has not been reported. The purpose of this study was to determine if the presence of *R. meliloti* around alfalfa roots alters the composition of root exudates either by increasing the amount of *nod* gene-inducing activity or by promoting exudation of phytoalexins.

MATERIALS AND METHODS

Plant Culture

"Moapa 69" alfalfa (*Medicago sativa* L.) seeds were scarified, surface-sterilized, allowed to imbibe, and grown under controlled conditions (Maxwell et al., 1989). *Rhizobium meliloti* strain 102F28 and *R. leguminosarum* bv *phaseoli* strain 4292 cells were cultured in tryptone-yeast extract (Beringer, 1974) and yeast extract-mannitol (Vincent, 1970) media, respectively, and rinsed cells were inoculated into hydroponic solution surrounding roots of 24-h-old seedlings to produce suspensions with $OD_{600} \approx 0.025$. Uninoculated controls received sterile nutrient solution (Maxwell et al., 1989). Solutions bathing roots, termed root exudates, were collected 2 to 9 d later (3–10 d after imbibition) and frozen at −80°C.

Tests for bacterial metabolism of exudate compounds used root exudate from 72-h-old uninoculated seedlings. *R. meliloti* 102F28 cells were suspended in the exudate ($OD_{600} \approx 0.040$) for 72 h and removed by centrifuging to supply exudate samples for HPLC analyses.

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Abbreviations: δ_H, chemical shift of proton; FAB, fast atom bombardment; 1H, one proton, etc.; H-1, proton on carbon one, etc.; Hz, Hertz; I_{max}, maximum β-galactosidase activity induced; I_{50}, concentration for half-maximum induction; MeOH, methanol; *s*, singlet; *d*, doublet; *dd*, double doublet; *m*, multiplet; *m/z*, mass/charge ratio.

Biological Activities

The *nod* gene-inducing capacity of root exudate and specific compounds was assayed as β-galactosidase activity (Miller, 1972) transcribed from a *nodC-lacZ* fusion with its associated *nodD1* gene on plasmid pRmM57 in *R. meliloti* strain 1021 (Mulligan and Long, 1985). Two other strains, JM57pRmJ30 and JM57pRmM137, which contained a *nodC-lacZ* fusion on pSym (JM57; Mulligan and Long, 1985) plus *nodD1* on multiple copies of pRmJ30 (Jacobs et al., 1985) or *nodD2* on multiple copies of pRmM137 (Fisher et al., 1988), were used to test for apparent activation of NodD1 and NodD2 proteins as reported previously (Hartwig et al., 1990a; Phillips et al., 1992). Activity of an uninduced control was subtracted. Strains were generously donated by Dr. S.R. Long, Stanford University (Stanford, CA).

Purification of Flavonoids from Root Exudates

Fresh or thawed root exudate was centrifuged at 6200*g* for 15 min and passed through 0.8- and 0.2-μm polycarbonate filters (Nuclepore Corp., Pleasanton, CA). Aliquots (50 mL) were adsorbed to 900-mg C_{18} Maxi-Clean cartridges (Alltech Associates, Inc., Deerfield, IL). Flavonoids from each cartridge were eluted with acetone, freeze-dried, and dissolved in 50% MeOH for HPLC analysis. Initial HPLC separations used a 250 × 4.6 mm Lichrosorb 5RP18 column (Phenomenex, Rancho Palos Verdes, CA), which was rinsed from 0 to 65 min with a linear gradient (0:99:1 to 99:0:1 [v:v:v] MeOH:water:acetic acid) and isocratically from 65 to 90 min with 99:1 MeOH:acetic acid at rate of 0.5 mL min^{-1}. Larger scale purification protocols used a 250 × 10 mm Lichrosorb 5RP18 semi-preparative column (Alltech Associates, Inc.) eluted at 2 mL min^{-1}. All eluates were monitored for absorbance at 260 to 400 nm with a photodiode array detector. Closely eluting compounds were purified by additional HPLC chromatography using appropriate MeOH concentrations.

Identification of Isoflavonoids

UV-visible spectral shift analyses (Mabry et al., 1970) were made with a Lambda 6 dual-beam spectrophotometer (Perkin-Elmer, Norwalk, CT). ^{1}H-NMR experiments were done in [U-^{2}H]MeOH on a GN-300 Omega NMR spectrometer (General Electric Co., Fremont, CA), and spectra were referenced to tetramethylsilane. FAB-MS data were collected with a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, UK) using positive ionization (xenon, 8 keV, 1 mA) with a VG Dynamic FAB probe. Samples were dissolved in MeOH and injected with 95:5 (v/v) water:glycerol at a flow rate of 5 μL min^{-1}.

Hydrolysates of purified flavonoids were prepared either by treating with 2.0 N HCl at 100°C for 1 h (acid or strong hydrolysis) or by incubating in 0.75 N NaOH at room temperature for 1 h under N_2 (alkaline or mild hydrolysis). In each case, the mixture was neutralized, freeze-dried, suspended in 50% MeOH, and centrifuged before repurifying by HPLC for further analyses.

Concentrations of isoflavonoids and their conjugates were estimated in ethanol using log ε = 4.44 at 250 nm for formononetin (Budavari, 1989) and log ε = 3.90 at 287 nm for medicarpin (Smith et al., 1971).

RESULTS

Inoculation Effects on Flavonoids in Root Exudate

Inoculating roots of 24-h-old alfalfa seedlings with either *R. meliloti* or *R.l.* bv *phaseoli* increased total *nod* gene-inducing activity of root exudates significantly within 48 h, and the effects remained evident for several days (Fig. 1). On day 5, the increase in *nod* gene-inducing activity over uninoculated controls was 200% for *R. meliloti* and 65% for *R.l.* bv *phaseoli*. Alfalfa root exudates were altered reproducibly in several experiments by both bacterial species, but all further studies were done with the normal alfalfa symbiont *R. meliloti*.

HPLC chromatograms of the flavonoid fraction from root exudates of plants exposed to *R. meliloti* were distinctly different from comparable eluents of uninoculated plants (Fig. 2). Peaks a, b, and c were easily identified by their UV-visible spectra, retention times, and relative *nod* gene-inducing activities as 4′,7-dihydroxyflavanone, 4,4′-dihydroxy-2′-methoxy-chalcone, and 4′,7-dihydroxyflavone, which were identified previously in exudates of uninoculated alfalfa seedlings (Maxwell et al., 1989). Peaks 1, 2, and 3, which were not evident in exudate from uninoculated plants, were chosen for further study because of either their *nod* gene-inducing activity (only peak 1) or their size (peaks 2 and 3). Although chromatograms from different experiments showed the same qualitative effects of rhizobial inoculation, differences in the heights of peaks 1, 2, and 3 from various experiments impaired efforts to quantify changes in the amounts of various flavonoids. The dangers associated with such attempts were emphasized by the fact that although peak 1 was often very small in chromatograms of root exudate (Fig. 2B), additional amounts of the compound were obtained by rinsing the inside surface of the root container with 50% MeOH. Compounds

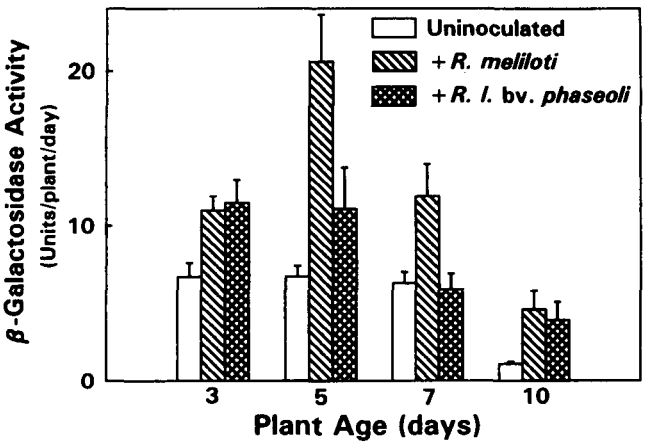


Figure 1. Effects of *R. meliloti* or *R. leguminosarum* bv *phaseoli* on *nod* gene-inducing activity in alfalfa root exudate. Induction of *nod* genes was assayed as β-galactosidase activity transcribed from a *nodC-lacZ* fusion in *R. meliloti* 1021pRmM57. Mean values + SE from five replicates are indicated.

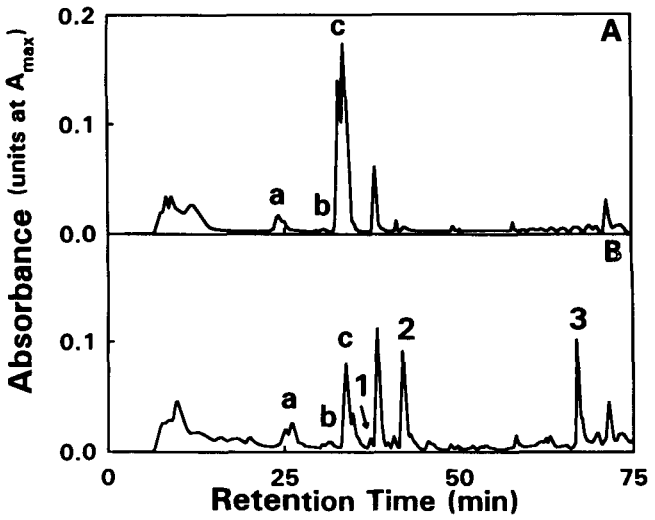


Figure 2. HPLC characteristics of the flavonoid fraction from root exudate (50 mL) of 3-d-old alfalfa seedlings collected from (A) uninoculated plants or (B) plants inoculated with *R. meliloti*. A_{max} (260–400 nm) was measured each second by a photodiode array detector. Labels a, b, and c indicate 4′,7-dihydroxyflavanone, 4,4′-dihydroxy-2′-methoxychalcone, and 4′,7-dihydroxyflavone. Peaks 1, 2, and 3 are identified here as a formononetin-7-*O*-(6″-*O*-malonylglycoside), a medicarpin-3-*O*-glycoside, and medicarpin.

in peaks 2 and 3 did not adhere to containers, and thus only small amounts of those molecules were available.

Compound Identifications

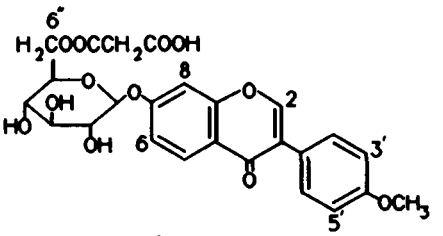
Absorbance measurements of peak 1 produced a UV-visible spectrum, A_{max}, MeOH at 203, 230, 249, 259, and 304 nm, similar to formononetin-7-*O*-glucoside (Mabry et al., 1970), and spectral shift experiments gave no evidence of free hydroxyl groups. The aglycone produced by acid hydrolysis of peak 1 gave UV-visible absorbance and shift results comparable to published data for formononetin (Mabry et al., 1970) and identical to authentic formononetin.

Unhydrolyzed peak 1 had the following proton resonances: δ_H ppm ([U-^{2}H]MeOH); 8.28 (1H, *s*, H-2), 8.16 (1H, *d*, J = 8.6 Hz, H-5), 7.50 (2H, *d*, J = 8.5 Hz, H-2′,6′), 7.29 (1H, *s*, H-8), 7.21 (1H, *dd*, J = 2.4, 8.6 Hz, H-6), 7.00 (2H, *d*, J = 8.5 Hz, H-3′,5′), 5.11 (1H, *d*, J = 3.7 Hz, H-1″), 4.55 (1H, *d*, J = 11 Hz, H-6″a), 4.26 (1H, *dd*, J = 6.7, 11 Hz, H-6″b), 3.84 (3H, *s*, OCH_3), 3.6 to 3.4 (4H, *m*, H-2″-5″). FAB-MS analyses produced major ions at m/z = 517, 431, and 269, which were consistent with MH^+ values calculated for a formononetin-malonylglycoside, a formononetin-glycoside, and formononetin, respectively.

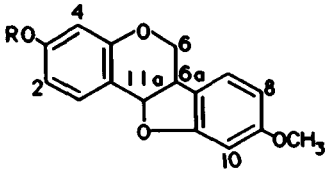
When peak 1 was hydrolyzed with alkali, the ^{1}H-NMR spectrum was comparable to the unhydrolyzed sample except that the H-6″a signal was shifted to 4.02 ppm (1H, *d*, J = 11 Hz) and the H-6″b signal shifted to 3.81 ppm (1H, *dd*, J = 6.1, 12 Hz). FAB-MS analyses of the alkali-treated sample showed major ions at m/z = 431 and 269, which were consistent with MH^+ values of formononetin-7-*O*-glucoside and formononetin. Upfield shifts in the 6″a and 6″b proton signals indicated that the malonyl group was attached at the C-6″ position (Matern et al., 1983; Kudou et al., 1991). Malonyl protons could not be detected in the solvent used in this study (Matern et al., 1983).

When peak 1 was hydrolyzed in acid, the ^{1}H-NMR spectrum showed no sugar signals and was identical to that of formononetin (Maxwell and Phillips, 1990). The four signals in the 7.0 to 6.85 ppm range could be assigned to individual protons after comparison with the unhydrolyzed sample: 6.97 (2H, *d*, J = 9.8 Hz, H-3′,5′), 6.92 (1H, *dd*, J = 2.4, 8.5 Hz, H-6), 6.84 (1H, *s*, H-8). Upfield shifts in the H-6 and H-8 proton signals were consistent with hydrolysis of a formononetin-7-*O*-glycoside linkage. FAB-MS analysis gave a molecular ion signal at m/z = 269, which corresponded to MH^+ of formononetin. ^{1}H-NMR and MS tests with authentic formononetin-7-*O*-glucoside and formononetin gave data identical to those recorded for alkali- and acid-hydrolysates of peak 1. On the basis of those results, it was concluded that peak 1 was a formononetin-7-*O*-(6″-*O*-malonylglycoside) (Fig. 3), which was previously identified in roots of six legumes (Köster et al., 1983). Only limited amounts of the compound were available, and identification of the sugar moiety was not feasible.

Absorbance measurements of peak 2 produced a UV-visible spectrum, A_{max}, MeOH at 206, 281, 286, and 308 nm, similar to medicarpin (Smith et al., 1971). Unhydrolyzed peak 2 had the following proton resonances: δ_H ppm ([U-^{2}H]MeOH); 7.39 (1H, *d*, J = 8.3 Hz, H-1), 7.16 (1H, *d*, J = 8.3 Hz, H-7), 6.78 (1H, *dd*, J = 2.3, 8.7 Hz, H-2), 6.62 (1H, *d*, J = 2.3 Hz, H-4), 6.43 (1H, *dd*, J = 2.3, 8.3 Hz, H-8), 6.37 (1H, *d*, J = 1.8 Hz, H-10), 5.49 (1H, *d*, J = 5.5 Hz, H-11a), 4.24 (1H, *d*, J = 5.5 Hz, H-6_{eq}), 3.86 (1H, *d*, J = 12 Hz, sugar H), 3.72 (3H, *s*, $-OCH_3$), 3.70 to 3.64 (1H, *m*, sugar H), 3.55 (1H, *d*, J = 6.0 Hz, H-6_{ax}), 3.52 (1H, *m*, H-6a), 3.45 to 3.35 (5H, *m*, sugar H). These values were consistent with those reported for medicarpin (Smith et al., 1971) and closely



Formononetin-7-*O*-(6″-*O*-malonylglucoside)



Medicarpin, R = H

Medicarpin-3-*O*-glycoside, R = sugar

Figure 3. Structures of formononetin-7-*O*-(6″-*O*-malonylglucoside), medicarpin-3-*O*-glycoside, and medicarpin.

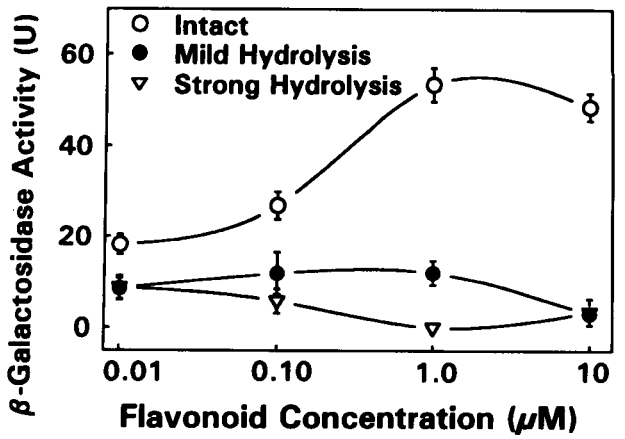


Figure 4. Induction of *nod* genes in *R. meliloti* 1021pRmM57 by formononetin-7-*O*-(6″-*O*-malonylglycoside) before (○) and after mild alkaline (●) or strong acid (▽) hydrolysis. Removal of malonate in akali and malonylglycoside in acid were confirmed by ^{1}H-NMR and MS analyses. Mean values ± SE are shown.

related molecules (Spencer et al., 1991), except for the downfield shift of the H-2 and H-4 signals, which indicated 3-*O*-glycosylation. Magnifying the spectrum suggested that additional details (e.g. a probable *dd* at 4.24 ppm) were comparable to published values for medicarpin, but the small amount of sample limited NMR resolution. FAB-MS analyses showed major ions at m/z = 433, 271, 270, and 269. Those ions are consistent with MH^+ values calculated for medicarpin glycoside, 433, and medicarpin, 271. The cluster of ions at m/z = 271, 270, and 269 was observed for authentic medicarpin analyzed under the same conditions. When the remainder of the sample purified from peak 2 was hydrolyzed in acid, the amount recovered was insufficient for further analysis.

Heights of HPLC peaks 2 and 3 (Fig. 2) were inversely related, and generally more of peak 2 was present than peak 3. As a result, insufficient amounts of peak 3 were recovered for NMR measurements. Peak 3 gave a UV absorbance spectrum, A_{max}, MeOH at 206, 280, 286, and 308 nm and a retention time on the HPLC in the standard gradient system that were identical to authentic medicarpin. Given the definite identification of peak 2 as a glycoside of medicarpin, these data supported a conclusion that peak 3 contained medicarpin aglycone.

nod Gene-Inducing Activity

Assays for *nod* gene induction in *R. meliloti* 1021pRmM57 showed that intact formononetin-7-*O*-(6″-*O*-malonylglycoside) produced an I_{50} value of approximately 300 nM, whereas both acidic and alkaline hydrolysis essentially inactivated the molecule (Fig. 4). Because hydrolyzed samples were analyzed by ^{1}H-NMR and FAB-MS before being assayed (data listed above), it was possible to conclude that removing either the malonyl or the malonylglycosyl portion of the molecule severely restricted activity of the intact compound. Those results were supported by direct assays with authentic formononetin, formononetin-7-*O*-glucoside, malonate, and formononetin-7-*O*-glucoside plus malonate, which gave no *nod* gene-inducing activity in *R. meliloti* 1021pRmM57. Control assays with the same strain showed that for luteolin I_{50} = 100 nM and I_{max} = 230 β-galactosidase units.

When the intact formononetin-7-*O*-(6″-*O*-malonylglycoside) was tested for *nod* gene induction in derivatives of *R. meliloti* strain JM57 that contained extra copies of *nodD1* or *nodD2* genes, the compound showed an apparent activation of both NodD1 and NodD2 regulatory proteins (Fig. 5). Luteolin (10 nM), a positive control for NodD1 activation, produced 40 units of β-galactosidase activity, and trigonelline (10 μM), a positive control for NodD2 activation, produced 51 units of β-galactosidase activity. Repeated assays confirmed that the compound was active with both *nodD* genes.

Effects of Bacterial Metabolism

Incubating root exudate from uninoculated plants with *R. meliloti* cells did not completely establish whether peaks 1, 2, and 3 (Fig. 2B) were plant or bacterial products. UV absorbance analyses of HPLC eluents from root exudates after incubating with bacteria away from the root never showed any evidence of either medicarpin molecule (peaks 2 and 3), but bacteria did affect the amounts and types of formononetin derivatives present in the exudate. HPLC peaks with formononetin-like UV spectra showed different retention times after incubation with bacteria. Those changes were consistent with modifications in hydrophobicity that would be produced by bacterial hydrolysis of malonyl or glycosyl groups. There was no evidence of increases in total formononetin-like compounds in the presence of bacteria. We conclude that bacteria do not synthesize medicarpin in the exudate but may modify formononetin conjugates.

DISCUSSION

Inoculating alfalfa roots with symbiotic *R. meliloti* cells clearly increased total *nod* gene-inducing activity in root

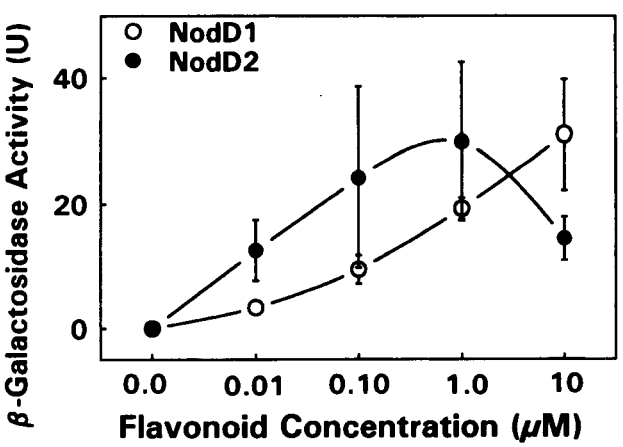


Figure 5. Induction of *nod* genes by a formononetin-7-*O*-(6″-*O*-malonylglycoside) purified from peak 1 (Fig. 2) and tested in *R. meliloti* strain JM57 containing extra copies of the regulatory genes *nodD1* (pRmJ30) or *nodD2* (pRmM137). Mean values ± SE are shown.

exudate (Fig. 1) and caused at least three additional compounds to appear (Fig. 2). Those three isoflavonoids (Fig. 3) are formed by many legumes. Isoflavonoid glycosides with 6″-*O*-malonylation have been isolated from at least 10 legumes (Beck and Knox, 1971; Köster et al., 1983; Kudou et al., 1991; Shibuya et al., 1991), including alfalfa (Kessman et al., 1990). Because roots of 6 legumes contained formononetin-7-*O*-(6″-*O*-malonylglucoside) (Köster et al., 1983) and all isoflavonoid glycosides in alfalfa roots contained Glc (Kessman et al., 1990), it is reasonable to expect that the small amounts of the *nod* gene-inducing formononetin-7-*O*-(6″-*O*-malonylglycoside) purified in this study contained Glc. Medicarpin is an isoflavonoid phytoalexin that can be elicited by pathogenic microbes in alfalfa (Smith et al., 1971) and *Vicia* (Hargreaves et al., 1976), but there is no report that microbial symbionts elicit the compound in legumes. Thus, our observation offers new insight into the parallel responses elicited in alfalfa by pathogens and a rhizobial symbiont. Growth of one strain of *R. meliloti* is not inhibited by medicarpin (Pankhurst and Biggs, 1980), so it may be possible that the symbiont gains a selective advantage over other microbes by stimulating the plant to release this phytoalexin.

Flavonoid changes in alfalfa root exudate after inoculation differed from those reported for *Vicia*. The *nod* gene-inducing activity in alfalfa root exudates was increased by the presence of both infective and noninfective rhizobia (Fig. 1), whereas only infective rhizobia had that effect on *Vicia* (van Brussel et al., 1990; Recourt et al., 1992). *Vicia* responded to the presence of infective rhizobia by releasing eight *nod* gene-inducing flavanones and chalcones but no isoflavonoid phytoalexins (Recourt et al., 1991). In contrast, only one new *nod* gene inducer was identified in alfalfa root exudate, but the phytoalexin medicarpin appeared in detectable quantities. The *Vicia* response to *Rhizobium* involved an increase in Phe ammonia lyase activity and chalcone synthase mRNA (Recourt et al., 1992). Whether the isoflavonoids identified in alfalfa root exudate originated from de novo synthesis or modification of existing compounds remains to be determined.

The 200% increase in total *nod* gene-inducing activity on day 5 for plants inoculated with *R. meliloti* (Fig. 1) probably cannot be attributed solely to the presence of formononetin-7-*O*-(6″-*O*-malonylglycoside). Comparisons indicate that this compound is a weaker *nod* gene inducer in *R. meliloti* 1021pRmM57 than luteolin. The 4,4′-dihydroxy-2′-methoxychalcone in root exudate of both inoculated and uninoculated alfalfa (Fig. 2, peak b) is a stronger inducer than luteolin (Maxwell et al., 1989), but 4′,7-dihydroxyflavone (Fig. 2, peak c) can inhibit activity of the chalcone (Hartwig et al., 1989). Thus, a decline in 4′,7-dihydroxyflavone after *R. meliloti* inoculation may have contributed to the increase in total *nod* gene-inducing activity of the crude exudate (Fig. 1). The decrease in 4′,7-dihydroxyflavone in the exudate is consistent with the observed increase in isoflavonoids because this compound is an important storage product in young alfalfa roots and a major portion of this compound exuded by alfalfa roots is closely linked to concurrent synthesis (Maxwell and Phillips, 1990). Presumably, redirecting carbon flow in that biosynthesis to isoflavonoids would decrease 4′,7-dihydroxyflavone in the exudate.

Formononetin-7-*O*-(6″-*O*-malonylglycoside) isolated in this study is only the second reported *nod* gene-inducing molecule that apparently activates both NodD1 and NodD2 regulatory proteins in *R. meliloti* (Fig. 5). The other compound, 4,4′-dihydroxy-2′-methoxychalcone, also induced *nod* genes in both *R. meliloti* strains JM57pRmJ30 and JM57pRmM137 (Hartwig et al., 1990b), but in the present case, it was the 6″-*O*-malonyl conjugate, not the flavonoid alone, that was responsible for activity (Fig. 4). The functions of these molecules in different regulatory pathways (i.e. NodD1 versus NodD2) remain to be defined (Fisher and Long, 1992). The ecological significance of formononetin-7-*O*-glycosides may be considerable because rhizosphere soil from alfalfa roots contains several closely related molecules with *nod* gene-inducing activity (León-Barrios et al., 1993). Indeed, it now appears that formononetin conjugates that coeluted with *nod* gene-inducing activity in alfalfa root extracts (Maxwell and Phillips, 1990) may have been related to compound 1 in this study and to the formononetin glycoside detected in rhizosphere soil extracts. Although we correctly concluded that formononetin was not an active *nod* gene inducer (Maxwell and Phillips, 1990), it may now be instructive to characterize those formononetin conjugates in root extracts more completely.

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