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# *Rhizobium nod* Gene Inducers Exuded Naturally from Roots of Common Bean (*Phaseolus vulgaris* L.)[1]

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## ABSTRACT

**Four compounds exuded from young roots of a black-seeded bean (*Phaseolus vulgaris* L., cv PI165426CS) induce transcription of *nod* genes in *Rhizobium leguminosarum* biovar *phaseoli*. The three most active *nod* gene inducers were identified by spectroscopic methods (ultraviolet/visible absorbance, proton nuclear magnetic resonance, and mass spectrometry) as being eriodictyol (5,7,3′,4′-tetrahydroxyflavanone), naringenin (5,7,4′-trihydroxyflavanone), and a 7-*O*-glycoside of genistein (5,7,4′-trihydroxyisoflavone). Comparisons with authentic standards verified the chemical structures of the aglycones and their capacity to induce β-galactosidase activity in *R. leguminosarum* strains containing *nodA-lacZ* or *nodC-lacZ* fusions controlled by *R. leguminosarum* biovar *phaseoli nodD* genes. Roots of 9-day-old seedlings released 42, 281, and 337 nanomoles per plant per day of genistein, eriodictyol, and naringenin, respectively. Genistein and naringenin induced higher maximum β-galactosidase activities and required lower concentrations for half-maximum induction than eriodictyol. Comparing the *nod* gene-inducing activity of seed rinses with root exudate from PI165426CS bean showed that root flavonoids were released at about 6% the rate of those from seeds on a molar basis, but on average the individual compounds from roots were approximately three times more active than *nod* gene inducers from seeds.**

An initial stage of root nodule formation on legumes is triggered by plant flavonoids that induce transcription of the common nodulation genes *nodABC* in *Rhizobium* bacteria (reviewed in ref. 15). This process requires the presence of an appropriate gene product from the regulatory *nodD* gene family in rhizobia, which interacts with the flavonoid. Numerous flavonoid structures have been reported as natural *nod* gene inducers from various legumes, including anthocyanidins (10), chalcones (19, 22), flavanones (5, 28), flavones (20, 23), flavonols (10), and isoflavones (12).

Although *nod*-inducing flavonoids have been reported from roots or seeds of diverse legumes, a detailed comparison of active molecules released separately from both seeds and roots of the same legume is available only for alfalfa (*Medicago sativa* L.) (8, 19). In alfalfa, organ-specific differences are evident in the amounts of *nod*-inducing activity released and in the types of flavonoids present. Functional differences between alfalfa root and seed flavonoids are supported by their capacities to activate various NodD proteins (9) and by their differing effects on rhizobial growth rate (7). Whether other legumes show similar differences between *nod* gene-inducing flavonoids released from seeds and roots is unknown.

The capacity of a flavonoid to interact with a *nodD* gene product is strongly affected by its molecular structure (15). The *nod* gene inducers released from alfalfa are structurally different (flavones from seeds [8, 20]; a chalcone, a flavanone, and a flavone from roots [19]), but they appear similar enough to fit easily into a single active site on the NodD protein. In contrast, reports indicate that *nod* genes in *R. leguminosarum* bv[3] *phaseoli* are induced by both the isoflavone genistein, which was tested as a commercially available compound (3, 26), and by anthocyanidin and flavonol molecules, which were identified as natural inducers from bean seeds (10). Although anthocyanidins and flavonols are rather similar in structure, they differ markedly from genistein. If the NodD protein of *R. leguminosarum* bv *phaseoli* is activated by both flavonols and genistein, then beans may release *nod* gene inducer molecules with even simpler structures, such as monocyclic phenolics. The purpose of this study was to identify the major *nod* gene inducers exuded by sterile bean roots and to compare them with those released from seeds (10).

## MATERIALS AND METHODS

### Preparation of Exudates

Black bean seeds (*Phaseolus vulgaris* L., cv PI165426CS) (4) were sterilized and imbibed in aerated sterile water as described previously (10). The rinse solution was collected and replaced periodically for 48 h to provide seed exudates. After 48 h, 17 germinating seeds were placed on a stainless steel screen (4 mesh, 0.035 gauge) over a 473-mL plastic container filled with 300 mL of sterile, aerated, N-free nutrient solution (19). The container was covered with sterile clear plastic as roots developed into the nutrient solution. Growth chamber conditions supplied a 12/12 h light/dark cycle, 25/22°C, 50% RH, and a photosynthetic photon flux density (400–700 nm) of 320 $\mu$E $m^{-2}$ $s^{-1}$. Solutions containing root exudates were changed and collected every 24 h for 8 d. The *nod*-inducing flavonoids identified in this study were purified

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[3] Abbreviations: bv, biovar; $\delta_H$, chemical shift of proton; 2H, two protons, etc.; C-1, carbon one, etc.; *d*, doublet; *s*, singlet; *dd*, double doublet; *t*, triplet; J, coupling constant; Hz, Hertz; $I_{max}$, the highest β-galactosidase activity; $I_{50}$, concentration for half-maximum induction.

from root exudates released by approximately 3400 plants between 216 and 240 h after imbibition. The solution from each container was centrifuged, filtered, and freeze dried (10).

### Purification and Identification of *nod* Gene Inducers

Compounds were separated for initial *nod*-inducing assays by resuspending dried material from three containers in 10 mL of methanol:water (1:1, v/v). A 500-$\mu$L aliquot of the mixture was injected into an HPLC system equipped with a reverse-phase C-18 column and separated on a 70-min linear gradient from 0:99:1 to 99:0:1 (v/v) methanol:water:acetic acid followed by 20 min of isocratic chromatography with 99:1 (v/v) methanol:acetic acid.

Compounds were isolated for identification by eluting filtered root exudate from C-18 Maxi Clean cartridges and partitioning against an equal volume of hexane to remove lipids as described previously (10). Compounds were separated by semi-preparative HPLC, collected, and analyzed spectroscopically (UV/visible absorbance shift analyses, $^1$H-NMR, and MS) (10). After tentative identifications, commercial standards were compared by spectroscopic analyses and co-chromatography. Standard interpretations were applied to UV/visible and $^1$H-NMR spectra (6, 17, 18) and to MS analyses (6, 16). Flavonoids present in root exudates were quantified by integrating HPLC peaks (10), and values were corrected for losses during purification by quantifying recovery of known amounts of eriodictyol added to root exudate.

### Biological Activities

The *nod*-inducing capacity of crude seed and root exudates, as well as specific compounds or fractions, was assayed as $\beta$-galactosidase activity transcribed from *nodA-lacZ* and *nodC-lacZ* plasmid fusions controlled by a *R. leguminosarum* bv *phaseoli nodD* gene (10). HPLC fractions were subdivided into portions equal to 1, 2, 5, 7.5, and 12.5% of the peak being tested and dried under vacuum for the assay. After compounds were identified, their *nod* gene-inducing activities were compared with HPLC-purified authentic commercial standards. Concentrations of flavonoids in assays were determined spectrophotometrically, using the following extinction coefficients (log $\epsilon$): 4.50 at 263 nm in ethanol for genistein (27), 4.24 at 292 nm in methanol for eriodictyol (27), and 4.23 at 288 nm in ethanol for naringenin (17). Flavonoid standards were purchased from Spectrum Chemical Manufacturing Corp. (Gardena, CA). Inducing activities of putative and authentic compounds were compared for statistically significant effects of concentration by analysis of variance.

## RESULTS

### Release of *nod* Gene-Inducing Activity

Root exudate fractionated on the HPLC contained four *nod* gene-inducing factors active in rhizobial strain RBL1283 (*nodA-lacZ*) (Fig. 1A). Peak 1 showed a very low level of *nod* gene-inducing activity in the 1% dilution, but peaks 2 through 4 gave considerable activity (Fig. 1B). These results were confirmed with $\beta$-galactosidase assays in *R. leguminosarum* bv *phaseoli*, strains containing *nodC-lacZ* fusions, CE-3

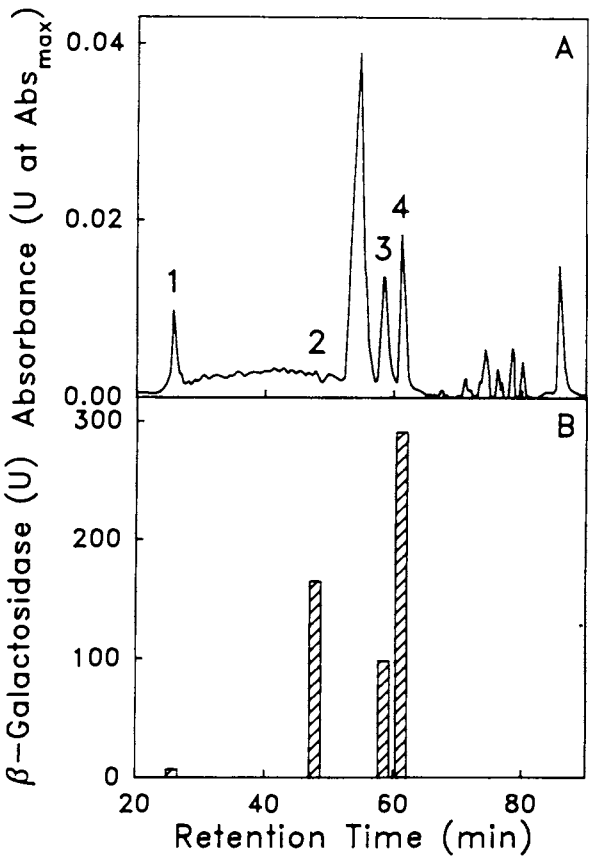


**Figure 1.** HPLC characteristics and *nod*-induction assays of bean root exudate. A, $A_{max}$ (230–400 nm) of exudates fractionated on a reverse-phase C-18 column. B, $\beta$-Galactosidase activity induced from *nodA-lacZ* in rhizobial strain RBL1283 containing *nodD* from *R. leguminosarum* bv *phaseoli*. Assays contained 1% of the corresponding HPLC fraction.

(pA87) and 4292 (pIJ1737, pIJ1730), described previously (10) (data not shown). Emphasis in this study was placed on peaks 2 through 4, which contained the most active *nod* gene inducers in all assays.

### Compound Identifications

#### *Compound 2*

Acid hydrolysis of peak 2 produced compound 2, which had UV/visible absorbance traits characteristic of an isoflavone (Table I). A bathochromic shift of band II with $CH_3ONa$ indicated the presence of free hydroxyl groups on the A ring, and their positions were deduced to be at C-7 and C-5 by the shifts in band II with $CH_3COONa$ and with $AlCl_3$/HCl, respectively. The absence of decomposition in $CH_3ONa$ is characteristic of a single 4′ hydroxyl on the B ring. These assignments of hydroxyls were supported by the following $^1$H-NMR data for compound 2: $\delta_H$ ppm ([U-$^2$H]methanol): 8.05 (1H, *s*, C-2), 7.34 (2H, *t*, J = 17.7 Hz, C-6′, C-2′), 6.83 (2H, *d*, J = 8.5 Hz, C-3′, C-5′), 6.32 (1H, *d*, J = 1.8 Hz, C-8), 6.20 (1H, *d*, J = 1.8 Hz, C-6). MS data supported a mol wt = 270 and showed a major fragment at *m/z* 152. Thus, the data

indicated a tentative identification of compound 2 as being genistein (5,7,4′-trihydroxyisoflavone) (Fig. 2). Peak 2 probably contains genistein glycosylated at the C-7 position because band II shows no shift in the presence of $CH_3COONa$ (Table I).

### *Compound 3*

Peak 3 was designated as compound 3 because acid hydrolysis did not release a smaller molecule, alter HPLC retention time, change the UV/visible traits, or affect *nod* gene-inducing activity. Absorbance traits typical for a 5,7-dihydroxyflavanone were evident in a 44-nm bathochromic shift of band II with $CH_3ONa$ (Table I). That interpretation was supported by a 35-nm shift in band II with $CH_3COONa$ (7-hydroxyl) and a 19-nm shift with $AlCl_3/HCl$ (5-hydroxyl). The $^1$H-NMR data identified the following resonances: $\delta_H$ ppm ([U-$^2$H]methanol): 6.83 (3H, *d*, J = 37.8 Hz, C-2′, C-6′, C-5′), 5.86 (2H, *dd*, J = 1.8, 6.1 Hz, C-8, C-6), 5.26 (1H, *dd*, J = 2.8, 12.8 Hz, C-2), 3.05 (1H, *dd*, J = 12.8, 17.1 Hz, C-3 trans), 2.67 (1H, *dd*, J = 3.0, 17.7 Hz, C-3 cis). MS data indicated a mol wt = 288 and gave fragments at *m/z* 287, 179, 153, 152, 136, and 123. Thus, the data supported a tentative identification of compound 3 as being eriodictyol (5,7,3′,4′-tetrahydroxyflavanone) (Fig. 2).

### *Compound 4*

Peak 4 was designated as compound 4 because acid hydrolysis did not release a smaller molecule, alter HPLC retention time, change the UV/visible traits, or affect *nod* gene-inducing activity. A 5,7-dihydroxyl substitution pattern in compound 4 was supported by UV/visible shift traits similar to compound 3 (Table I). The $^1$H-NMR data identified the following resonances: $\delta_H$ ppm ([U-$^2$H]methanol): 7.30 (2H, *d*, J = 7.9 Hz, C-6′, C-2′), 6.80 (2H, *d*, J = 8.5 Hz, C-3′, C-5′), 5.86 (2H, *d*, J = 3.0 Hz, C-8, C-6), 5.33 (1H, *dd*, J = 1.8, 12.8 Hz, C-2), 3.11 (1H, *dd*, J = 3.6, 8.5 Hz, C-3 trans), 2.67 (1H, *dd*, J = 3.0, 17.1 Hz, C-3 cis). MS data indicated a mol wt = 272 with fragments at *m/z* 153, 152, 120, and 107. Thus, the data supported a tentative identification of compound 4 as being naringenin (5,7,4′-trihydroxyflavanone) (Fig. 2).

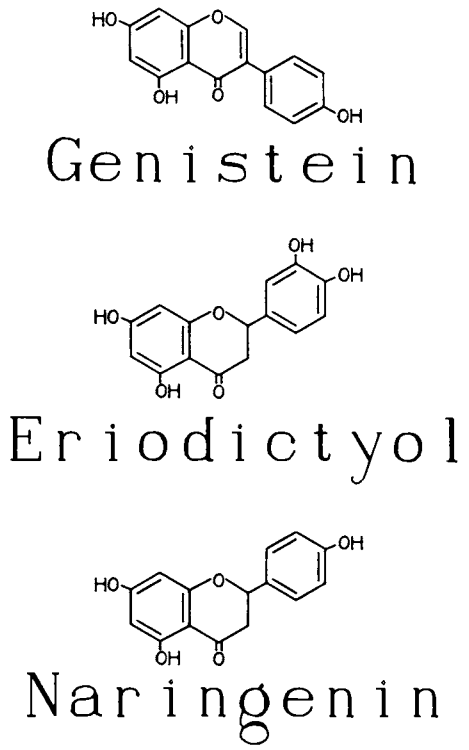


**Figure 2.** Structures of *nod*-inducing flavonoids exuded from bean roots. Genistein is released as a glycoside; eriodictyol and naringenin are exuded as aglycones.

### Verification of Identifications

Spectroscopic analyses (UV/visible, $^1$H-NMR, and MS) of authentic genistein, eriodictyol, and naringenin matched results obtained with compounds 2, 3, and 4, respectively. Furthermore, co-chromatography of standards with the root exudate compounds confirmed the putative identifications.

### *nod*-Inducing Activities of Compounds

The *nod* gene-inducing activity of authentic and putative samples of compounds 2 through 4 showed similar concentration dependencies after repurification by HPLC immedi-

**Table I.** *UV/Visible Absorbance Maxima (nm) of nod Gene Inducers in Bean Root Exudate*

Standard reagents and procedures were used (17).

| Compound[a] | Methanol | Shift Reagent | | | | |
|---|---|---|---|---|---|---|
| | | $CH_3ONa$ | $AlCl_3$ | $AlCl_3/HCl$ | $CH_3COONa$ | $CH_3COONa/H_3BO_3$ |
| 2G | 261, 329sh[b] | 271, 355sh | 272, 309sh, 374 | 272, 309sh, 373 | 262, 330sh | 261, 329sh |
| 2A | 261, 328sh | 274, 326sh | 273, 308sh, 371 | 273, 309sh, 371 | 272, 327 | 262, 335sh |
| 3A | 289, 323sh | 245, 323 | 309, 376 | 308, 372 | 289sh, 324 | 290, 334sh |
| 4A | 289, 327sh | 245, 323 | 311, 374 | 310, 371 | 284sh, 323 | 289, 331sh |

[a] G, glycoside; A, aglycone. [b] sh, spectral shoulder.

**Table II.** *Comparison of nod-Inducing Activity for Putative Flavonoid Aglycones from Bean Roots and Authentic Standards*

Mean values are from three replicates assayed in rhizobial strain RBL1283.

| Flavonoid Concentration | Genistein | | Eriodictyol | | Naringenin | |
|---|---|---|---|---|---|---|
| | Putative | Authentic | Putative | Authentic | Putative | Authentic |
| *nM* | *units of β-galactosidase* | | | | | |
| 10 | 45 | 51 | 0 | 0 | 23 | 17 |
| 20 | 341 | 298 | 81 | 67 | 242 | 222 |
| 50 | 743 | 863 | 176 | 167 | 489 | 450 |
| 100 | 1390 | 1320 | 216 | 220 | 830 | 799 |
| 200 | 2290 | 2190 | 328 | 311 | 1210 | 1200 |
| 500 | 2900 | 2640 | 470 | 442 | 1530 | 1490 |
| 1,000 | 3010 | 3120 | 600 | 586 | 1960 | 2020 |
| 2,000 | 3960 | 3670 | 711 | 733 | 2460 | 2280 |
| 5,000 | 4000 | 3870 | 814 | 825 | 2480 | 2490 |
| 7,500 | 4120 | 4020 | 821 | 837 | 2500 | 2500 |
| 10,000 | 4180 | 4210 | 830 | 822 | 2410 | 2510 |
| LSD 0.05 | 440 | | 69 | | 341 | |

ately before assaying (Table II). Comparisons among the flavonoids in root exudates showed that genistein had the highest $I_{max}$ and the lowest $I_{50}$. No significant differences ($P \leq 0.05$) in $I_{max}$ values were detected for compound 2 (genistein) and unhydrolyzed peak 2 (genistein-7-*O*-glycoside). The $I_{50}$ value for the glycoside, however, was slightly lower (120 *versus* 151 nM). Mean $I_{50}$ values (nM) were genistein, 151; naringenin, 187; and eriodictyol, 270. Compounds 3 and 4, which are released as aglycones, induced similar amounts of β-galactosidase activity before and after hydrolysis.

### Amounts of Compounds

Roots released total *nod* gene-inducing activity at about half the rate of seeds tested during the first 6 h of imbibition (Fig. 3). Using a 90% recovery rate measured as a correction factor for eriodictyol added to root exudate, HPLC integrations showed the following amounts of individual *nod*-inducing flavonoids exuded from bean roots between 216 and 240 h after imbibition (nmol plant$^{-1}$ day$^{-1}$): genistein, 42; eriodictyol, 281; and naringenin, 337. The three root compounds showed an average $I_{50}$ of 200 nM with a mean $I_{max}$ of 2480 units (Fig. 4), whereas three flavonols and three anthocyanidins identified from seeds (10) gave a mean $I_{50}$ of 670 nM and an average $I_{max}$ of 1030 units.

## DISCUSSION

Data from this study identify genistein, eriodictyol, and naringenin as being three *nod* gene-inducing flavonoids ex-

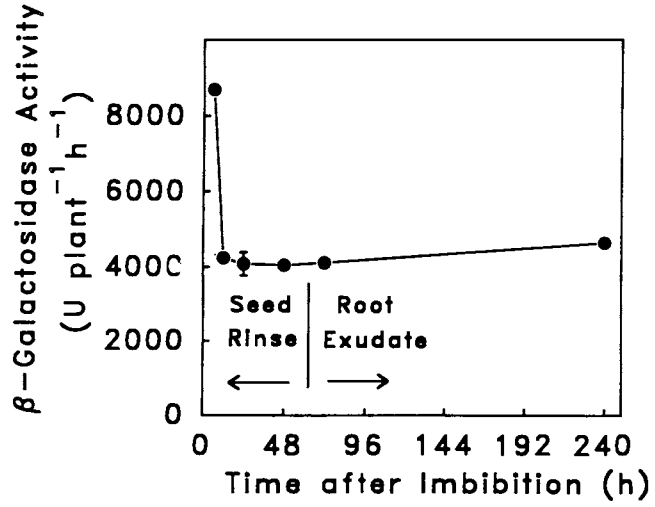


**Figure 3.** Release of *nod* gene-inducing activity from seeds and roots of bean line PI165426CS. Mean ± SE values are from six replicates assayed for β-galactosidase activity induced from *nodA-lacZ* controlled by *nodD* from *R. leguminosarum* bv *phaseoli* in rhizobial strain RBL1283.

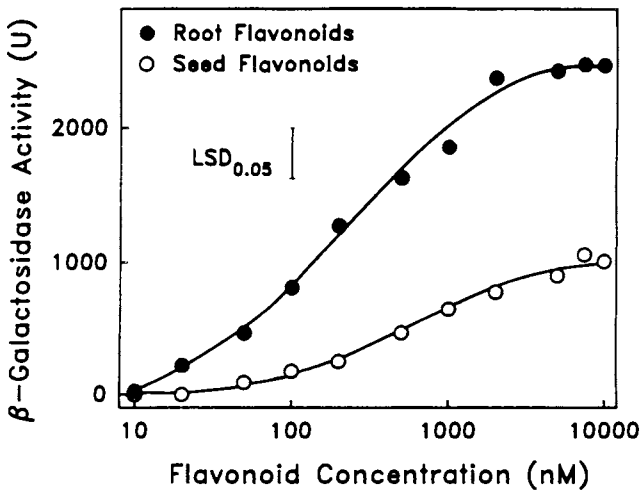


**Figure 4.** Induction of *nod* genes in rhizobial strain RBL1283 by flavonoids released from bean seeds and roots. Values for flavonoids identified as natural products from bean seeds (10) are means of six aglycones (delphinidin, petunidin, malvidin, myricetin, quercetin, and kaempferol). Root flavonoids are means of three *nod* gene inducers in root exudate identified in this study (genistein, eriodictyol, and naringenin). All means are from three replicates.

uded naturally from sterile bean roots. These aglycones are common plant flavonoids (2, 11), but they were not identified in bean seed rinses (10). Eriodictyol (5) and genistein (12) have been implicated previously as natural *nod* gene inducers released under sterile conditions from pea and soybean, respectively whereas naringenin has been used as a potent inducer in *R. leguminosarum* bv *phaseoli* (3, 26), its presence in natural bean exudates has not been reported.

Although the aglycones identified in this study were not observed in bean seed rinses (10), they share a common hydroxylation pattern with substitution at the C-4′, C-5, and C-7 positions. Whereas the 4′-hydroxyl may contribute to activity, it clearly is not required, because chrysin (5,7-dihydroxyflavone) and galangin (3,5,7-trihydroxyflavone) induce *nod-lacZ* transcription in *R. leguminosarum* bv *phaseoli* (3). Moreover, the aryl shift of the B ring from the C-2 to the C-3 position in genistein (Fig. 2) places the 4′-hydroxyl of that compound in a much different position than for any of the other natural *nod* gene inducers from bean. It seems reasonable, therefore, that hydroxylation similarities in these compounds may reflect their common biosynthetic pathways (24) rather than uniform requirements for interacting with the *nodD* gene product.

One notable feature of bean is the large variety of flavonoid structures that is released as natural *nod* gene inducers. To the anthocyanins and flavonols released from seeds (10), one must now add the flavanones and isoflavone identified in root exudate (Fig. 2). These diverse compounds all were active with both the *nodA-lacZ* and the *nodC-lacZ* fusions tested in this study, even though *nodA* and *nodC* are transcribed separately in *R. leguminosarum* bv *phaseoli* (26). Natural *nod* gene inducers released from alfalfa seeds and roots show clear structural differences (5-hydroxy from seeds *versus* 5-deoxy from roots; 8, 19), but no such distinction is evident for bean. One cannot make definitive statements about interactions between flavonoids and *nodD* gene products until those purified proteins are available for binding studies, but the diversity of active flavonoid structures released from bean and their uniformly higher $I_{50}$ values suggest that NodD proteins from *R. leguminosarum* bv *phaseoli* are much less specific for flavonoids than their counterparts in *R. meliloti* (8, 10, 19). Despite this possibly lower level of specificity in the *R. leguminosarum* bv *phaseoli* NodD proteins, no *nod* gene-inducing monocyclic phenolics, comparable to those isolated from wheat root extracts (14), were identified in bean root exudates. The suggestion that structural differences in bean *nod* gene inducers may be related to their capacity to inhibit *nod* gene expression in other rhizobia (10) is supported by effects of bean root flavonoids on other rhizobial species (*e.g.* genistein inhibits *nod* gene induction in *R. leguminosarum* bvs *viceae* and *trifolii* [5]; naringenin inhibits *nod* gene induction in *R. meliloti* [21] and some *Bradyrhizobium japonicum* strains [13]).

Data on the amount of flavonoids released from germinating bean seeds (10) and roots (Fig. 2) also offer an interesting comparison with recently described *nod* gene-inducing flavonoids that are released from alfalfa (8, 19, 20). Bean seeds used for these studies were about 80 times larger than alfalfa seeds, but the rates of discharge for *nod* gene inducers identified from bean are 6000 to 7000 times higher than the comparable values for alfalfa (bean: seeds, 497 nmol $seed^{-1}$ $h^{-1}$ [10], roots, 28 nmol $plant^{-1} \cdot h^{-1}$; alfalfa: seeds, 82 pmol $seed^{-1}$ $h^{-1}$ [8], roots, 4 pmol $plant^{-1}$ $h^{-1}$ [19]). These numerical comparisons must be tempered with the knowledge that they are based on a limited number of plant and bacterial genotypes, but such large differences in total amount of flavonoids might be explained by several factors. First, beans may release larger quantities of flavonoids than alfalfa because the bean *nod* gene inducers have higher $I_{50}$ values than those of alfalfa when tested in their respective rhizobia (*e.g.* genistein $I_{50}$ = 150 nM; 4,4′-dihydroxy-2′-methoxychalcone $I_{50}$ = 1 nM [19]). Second, due to its annual life history, bean may benefit more from an early establishment of root nodules than the perennial alfalfa.

Overall, bean and alfalfa root exudates contain lesser quantities of *nod* gene-inducing flavonoids than seed rinses (Fig. 3) (8, 19), but the average activity of the individual root compounds is greater than that of *nod* gene inducers from seed rinses (Fig. 4) (8, 19). This fact suggests that soil surrounding these organs should be viewed as separate ecochemical zones in terms of both the amounts and structures of flavonoids. With the identity of *nod* gene-inducing flavonoids established for seeds (10) and roots (Fig. 2), other biological effects of these natural compounds can be examined. Although it has been reported that *R. leguminosarum* bv *phaseoli* shows a positive chemotaxis toward luteolin (1), tests can now be conducted with the flavonoids that are actually present in the seed and root zone of bean. Similarly, bean flavonoids can be tested for effects comparable to those demonstrated for alfalfa flavonoids on growth of rhizobia (7) and germination of *Glomus* (25). Testing these possible roles of naturally released bean flavonoids can help define ecochemical differences between seed and root zones.

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