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Anthocyanidins and Flavonols, Major *nod* Gene Inducers from Seeds of a Black-Seeded Common Bean (*Phaseolus vulgaris* L.)[1]

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ABSTRACT

Eleven compounds released from germinating seeds of a black-seeded bean (*Phaseolus vulgaris* L., cv PI165426CS) induce transcription of *nod* genes in *Rhizobium leguminosarum* biovar *phaseoli*. Aglycones from 10 of those compounds were identified by spectroscopic methods (ultraviolet/visible, proton nuclear magnetic resonance, and mass spectroscopy), and their biological activities were demonstrated by induction of β-galactosidase activity in *R. leguminosarum* strains containing *nodA-lacZ* or *nodC-lacZ* fusions controlled by *R. leguminosarum* biovar *phaseoli nodD* genes. By making comparisons with authentic standards, the chemical structures for aglycones from the 10 molecules were confirmed as being anthocyanidins (delphinidin, petunidin, and malvidin) and flavonols (myricetin, quercetin, and kaempferol). All anthocyanidins and flavonols had 3-*O*-glycosylation and free hydroxyl groups at the 4′, 5, and 7 positions. Hydrolysis experiments showed that the mean concentration required for half-maximum *nod* gene induction (I_{50}) by the 10 glycosides was about half that of the corresponding aglycones. The mean I_{50} value for the three anthocyanidins (360 nanomolar) was less ($P \leq 0.05$) than that of the three flavonol aglycones (980 nanomolar). Each seed released approximately 2500 nanomoles of anthocyanidin and 450 nanomoles of flavonol *nod* gene inducers in conjugated forms during the first 6 hours of imbibition. Based on amounts and activities of the compounds released, anthocyanins contributed approximately 10-fold more total *nod*-inducing activity than flavonol glycosides. These anthocyanidins from bean seeds represent the first *nod*-inducing compounds identified from that group of flavonoids.

Root nodule formation in legumes requires expression of *nod* genes in *Rhizobium* and *Bradyrhizobium* bacteria (reviewed in ref. 22). Transcription of *nodABC* is induced through the cooperative action of the protein product of the *nodD* gene and components of root and seed exudates. Particular flavonoids have been identified as natural *nod*-inducing factors for leguminous hosts of *R. meliloti* (13, 26, 29), *B. japonicum* (18, 33), *R. leguminosarum* bv[3] *viceae* (8, 31, 40), *R. leguminosarum* bv *trifolii* (32), and the broad-host-range *Rhizobium* NGR234 (2). Some commercially available flavonoids induce *nod* genes in *R. leguminosarum* bv *phaseoli* (4), but no naturally occurring *nod* inducers have been reported from common bean.

A detailed description of natural *nod* gene inducers released by alfalfa (*Medicago sativa* L.) is available (13, 26, 29). To assess whether the pattern of flavonoid release is unique to that species, a comparative study of *nod* gene inducers in another legume is needed. In this regard, an analysis of flavonoids released by common bean (*Phaseolus vulgaris* L.) is interesting for several reasons. First, like alfalfa, it is nodulated by a *Rhizobium* with a complex family of regulatory *nodD* genes that includes three alleles (4). Second, major differences in life history and site of origin between perennial alfalfa and annual bean may maximize the potential for finding different patterns of flavonoid synthesis, release, and function. Third, poor nodulation frequently is observed in bean (28), and understanding *nod*-inducing flavonoids exuded by that plant may help solve this agronomic problem. The purpose of the present study was to identify *nod*-inducing flavonoids released by common bean seeds as a first step toward assessing if the availability of flavonoids can limit nodulation in this legume, as they may in alfalfa (14, 16).

MATERIALS AND METHODS

Preparation of Exudates

Black bean seeds (*Phaseolus vulgaris* L., cv PI165426CS) (5) were sterilized for 2 min in 95% ethanol followed by 2 min in 50% commercial bleach and then washed 10 times with sterile water. One hundred seeds (about 20 g) imbibed in 40 mL of aerated sterile water for 6 h in the dark at 27°C yielded 12 to 14 mL of solution, which was termed seed rinse or exudate for this study.

Purification of *nod* Gene Inducers

Small-scale studies of biological activity and quantification of flavonoids were done with 250 μL aliquots of seed exudate

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[3] Abbreviations: bv, biovar; δ_H, chemical shift of proton; 2H, two protons, etc.; C-1, carbon one, etc.; *d*, doublet; *s*, singlet; J, coupling constant; Hz, Hertz; I_{50}, concentration required for induction of 50% of the maximum β-galactosidase activity.

diluted 1:1 with methanol. The resulting 500 μL sample was injected into a Waters HPLC system (Millipore Corp., Milford, MA) equipped with a 50 × 10 mm Lichrosorb 5RP18 guard column (Phenomex, Torrance, CA) and a 250 × 10 mm Maxsil C-18, 5-μm particle size, semipreparative reverse-phase column (Phenomex). The column was eluted at 2.0 mL min^{-1} with a 90-min linear gradient from 0:99:1 to 99:0:1 (v/v) methanol:water:acetic acid. Compounds were monitored with a Waters 990 photodiode array detector that measured absorbance (230–560 nm) every second. Eluent fractions were collected every minute, subdivided into replicate aliquots, and dried under vacuum for assays.

Large-scale purification of active fractions was done with seed exudate that was centrifuged at 6200*g* for 15 min, filtered through 0.8- and 0.4-μm polycarbonate filters (Nucleopore Corp., Pleasanton, CA), and stored at −80°C prior to use. Thawed filtrate was adsorbed to a 900-mg C-18 Maxi-Clean cartridge (Alltech Associates, Inc., Deerfield, IL) using 3 mL of solution per cartridge. To prevent precipitation of poorly soluble pigments, flavonoids were eluted from cartridges with 5-mL aliquots of 0, 30, 50, 80, and 100% (v/v) methanol:water rinses, then the eluates were recombined and freeze-dried. A sample corresponding to 3 mL of seed exudate was resuspended in 500 μL of 50% methanol, partitioned against hexane (1:1, v/v) to remove remaining lipid, and chromatographed on the standard linear HPLC gradient over 200 min for better separation of the compounds. Eluent peaks of interest were characterized by retention times and UV/visible spectra from the photodiode array detector and freeze-dried. A 0.4-mg portion of each compound was hydrolyzed in 2 N HCl at 100°C for 40 min (12), freeze-dried, and cleaned on the HPLC for ^{1}H-NMR and UV/visible shift analyses. A subsample of each peak was cleaned again in the HPLC for the MS analyses after regenerating the column with hot water, methanol, acetonitrile, and tetrahydrofuran.

Biological Activities

The *nod*-inducing capacity of HPLC fractions was assayed as β-galactosidase activity (27) from *nod-lacZ* fusions under the control of *nodD* genes from *R. leguminosarum* bv *phaseoli* using techniques described previously (26), except bacteria were grown on yeast-mannitol medium (37). Controls measuring the separate effects of bacteria and inducers were run in all assays. In every case reported here, controls containing only bacteria produced a higher absorbance, which was subtracted as the background value. The initial bacterial strain used, RBL1283, was generously supplied by Drs. B. J. J. Lugtenberg and R. J. H. Okker (Leiden University). This strain was produced from *R. leguminosarum* bv *trifolii* RCR5, which, after having been cured of its symbiotic plasmid, was given *pSym9*-Tn*5* (a symbiotic plasmid from *R. leguminosarum* bv *phaseoli* LPR9001) and pMP154 (containing a *nodA-lacZ* fusion) (35). Subsequently, *nod*-inducing activities of compounds were verified with two strains containing *nodC-lacZ* fusions: *R. leguminosarum* bv *phaseoli* CE-3(pA87) (kindly provided by Dr. F. Sanchez, UNAM, Cuernavaca, Mexico) and *R. leguminosarum* bv *phaseoli* 4292 containing pIJ1737 (*nodC-lacZ*) and pIJ1730 (extra *nodD1*) (4), a generous gift from Dr. A. W. B. Johnston (University of East Anglia).

Initial assays for *nod*-inducing activity were done with samples containing 1, 2, 5, 7.5, or 12.5% of HPLC eluent fractions collected each minute. When authentic standards were assayed, they were purified by HPLC before use. Flavonoid concentrations in assays were calculated spectrophotometrically relative to a final volume of 475 μL with known extinction coefficients (log ε): delphinidin, 4.49 at 555 nm in ethanol-0.01% HCl (12); delphinidin glucoside, 4.46 at 543 nm in methanol-1% HCl (delphinidin-3-*O*-monoglucoside) (11); petunidin, 4.54 at 550 nm in the organic phase of 10% phosphoric acid:phenol:toluene (2:2:1) (34); petunidin glycoside, 4.11 at 546 nm in methanol-0.1% HCl (petunidin-3-*O*-monoglucoside) (11); malvidin, 4.50 at 547 nm in methanol-0.01% HCl (12); malvidin glucoside, 4.45 at 520 nm in methanol-0.1 N HCl (malvidin-3-*O*-monoglucoside) (10); myricetin, 4.29 at 378 nm in ethanol (24); myricetin glycoside, 4.20 at 363 nm in ethanol (myricetin-3-*O*-galactoside) (15); quercetin, 4.32 at 373 nm in ethanol (38); quercetin glycoside, 4.24 at 360 nm in ethanol (quercetin-3-*O*-arabinose) (24); kaempferol, 4.20 at 273 nm in ethanol (38). A log ε of 4.20 at 273 nm in ethanol was assumed for the kaempferol glycosides.

Identification of *nod* Gene Inducers

UV/visible spectra were measured in a Lambda 6 dual beam spectrophotometer (Perkin Elmer, Norwalk, CT). Spectral shift experiments for flavonols (peaks 7–11) were done with standard procedures (24, 25). UV/visible spectra for anthocyanins (peaks 2–6) were obtained in methanol-0.01% HCl. The shift in $AlCl_3$ was measured with the compound dissolved in methanol at pH 3.0 (12).

One-dimensional ^{1}H-NMR analyses were done on a GN-300 Omega NMR spectrometer (General Electric Co., Fremont, CA). Anthocyanins and anthocyanidins were dissolved in [U-^{2}H]DMSO-[U-^{2}H]HCl, while other compounds were dissolved in [U-^{2}H]methanol. The solvent peak was used as an internal standard (DMSO, 2.49 ppm; methanol, 3.30 ppm).

MS data were collected on a ZAB-HS-2F MS (VG Analytical, Wythenshawe, UK) with positive and negative ionization (xenon, 8 keV, 1 mA) or electron ionization on a Trio-2 MS (VG Masslab, Altrincham, UK) using a probe to introduce the samples.

HPLC co-chromatography of authentic standards followed procedures described above, except that a 70-min linear gradient was used. Authentic delphinidin, malvidin, myricetin, quercetin, kaempferol, and quercetin-3-*O*-monogalactoside were purchased from Spectrum Chemical Manufacturing Corp. (Gardena, CA). Dr. G. Hrazdina (Cornell University) kindly provided malvidin-3-*O*-monoglucoside and petunidin-3-*O*-monoglucoside. Petunidin-3-*O*-monoglucoside was hydrolyzed to produce petunidin.

Quantification of Flavonoids

Amounts of *nod* inducers in seed rinses were estimated by HPLC with known quantities of authentic standards by ap-

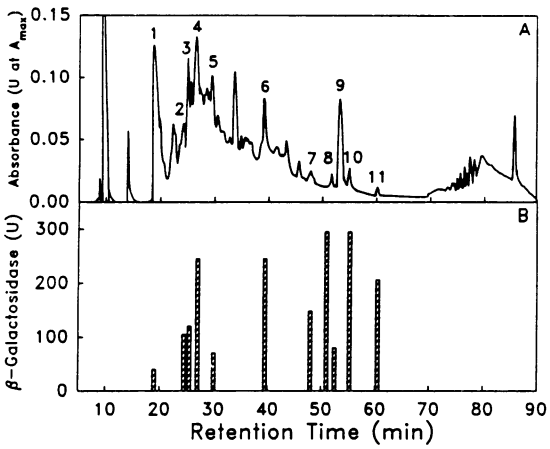

Figure 1. HPLC characteristics and *nod*-induction assays of bean seed exudate released during the first 6 h of imbibition by the black-seeded line PI165426CS. A, A_{max} (230–560 nm) of exudates fractionated in a methanol-water gradient on a reverse-phase C_{18} column. B, β-Galactosidase activity induced from a *nodA-lacZ* fusion controlled by *nodD* from *R. leguminosarum* bv *phaseoli* in rhizobial strain RBL1283. Assays contained 1% of the corresponding HPLC fraction.

plying the integrator function with the Waters 990 software package. Correction factors for flavonoid recovery were determined by adding known amounts of malvidin-3-*O*-monoglucoside and quercetin-3-*O*-monogalactoside to seeds during imbibition and purifying flavonoids with the normal procedures.

RESULTS

Release of *nod*-Gene Inducers

Eleven HPLC fractions from the 6-h seed rinse induced significant β-galactosidase activity from the *nodA-lacZ* fusion in rhizobial strain RBL1283 at the lowest dilution tested (1% of the eluent peak; Fig. 1) and produced more activity with increasing concentrations of the eluent peak (data not shown). The same fractions induced β-galactosidase activity in *R. leguminosarum* bv *phaseoli* strains containing *nodC-lacZ* fusions, CE-3 (pA87) and 4292 (pIJ1737, pIJ1730) (data not shown). Three experiments with all strains showed similar responses in each strain for all 11 compounds.

Individual *nod* Inducers

Peak 1

No compound present in peak 1 was identified. This *nod*-inducing factor, which is highly soluble in both water and methanol, has UV/visible absorbance spectra and ^{1}H-NMR resonances consistent with an isoflavone (data not shown). Extensive hydrolysis (4 h) released no aglycone.

Anthocyanidins (Compounds 2–6)

Analyses of compounds purified from peaks 2 through 6 produced data consistent with published characteristics of anthocyanins (unhydrolyzed sample) and anthocyanidins (hydrolyzed fraction) for UV/visible absorbances (10, 12, 39), ^{1}H-NMR spectra (36), and MS molecular ions (3). Unhydrolyzed compounds showed no absorbance peak characteristic of acyl groups (310–335 nm), and $A_{acyl}/A_{vis\ max}$ ratios less than 0.20 were recorded for all anthocyanins (data not shown). The presence of an *O*-glycoside linkage in all compounds was indicated by their easy hydrolysis in acid. Attachment of sugars at only the C-3 position (R_1, Fig. 2A) was supported by (a) $A_{440}/A_{vis\ max}$ ratios of 0.18 to 0.20 (data not shown); (b) $A_{UV\ max}/A_{vis\ max}$ ratios of 0.49 to 0.56 (data not shown); and (c) band II maxima at 275 to 276 nm (Table I). Reported values for C-5 glycosides or C-3,5-diglycosides of delphinidin, petunidin, and malvidin are considerably lower for each of these three parameters (10, 12, 39).

Compound 2. An identification of the aglycone produced by hydrolysis of peak 2 as being the anthocyanidin delphinidin (3,5,7,3′,4′,5′-hexahydroxyflavylium) (Table II) was supported by (a) MS data indicating a mol wt of 303; (b) ^{1}H-NMR resonances (Table I) matching authentic delphinidin; (c) a bathochromic shift of 24 nm of band I in the presence of aluminum ion (Table I), which indicates *o*-dihydroxyl groups in the B ring; (d) UV_{max} and vis_{max} absorbances (Table I) matching authentic delphinidin; and (e) co-chromatography with authentic delphinidin.

Compounds 3 and 5. An identification of the aglycone produced by hydrolysis of peaks 3 and 5 as being the anthocyanidin petunidin (3,5,7,4′,5′-pentahydroxy-3′-methoxyflavylium) (Table II) was supported by (a) MS data indicating a mol wt of 317; (b) ^{1}H-NMR resonances (Table I) matching authentic petunidin; (c) a bathochromic shift of 21 nm of band I in the presence of aluminum ion (Table I), which indicates *o*-dihydroxyl groups in the B ring; (d) UV_{max} and

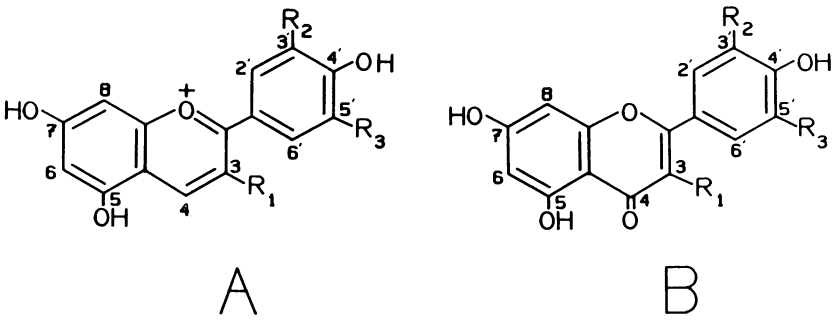

Figure 2. Aglycone structures of *nod*-inducing flavonoids released by a black-seeded bean line during imbibition. A, Anthocyanidins. B, Flavonols. R_1, R_2, and R_3 substituents for 10 compounds are listed in Table II.

Table I. *UV/Visible and ^{1}H-NMR Data for Compounds from Peaks 2 through 6 in Figure 1*

Peak[a]	Color	UV/Visible[b]			NMR Protons[c]						
		Absorbance		$AlCl_3$ shift	H-4	H-6	H-8	H-2′	H-6′	OMe-3′	OMe-5′
		UV_{max}	Vis_{max}								
		nm		*nm*				*ppm*			
2A	purple	277	548	24	8.79	6.81	6.93	7.75	7.75		
2G	purple	276	536	24							
3A	purple	276	547	21	8.86	6.86	7.01	7.77	7.87	3.85	
3G	purple	276	534	20							
4A	red-purple	275	545	0	8.92	6.91	7.12	7.94	7.94	3.91	3.91
4G	purple	276	533	0							
5A	purple	276	547	21	8.78	7.12	7.18	7.75	7.94	3.90	
5G	purple	276	535	20							
6A	red-purple	275	545	0	8.92	7.12	7.19	7.95	7.95	3.91	3.91
6G	purple	276	534	0							

[a] A, aglycone; G, glycoside. [b] Measured in methanol-0.01% HCl except for $AlCl_3$, which was in methanol, pH 3.0. [c] Measured in [U-^{2}H]DMSO-[U-^{2}H]HCl.

vis_{max} absorbances (Table I) matching authentic petunidin; and (e) co-chromatography with authentic petunidin. The split in ^{1}H-NMR resonances assigned to H-2′ and H-6′ for compounds 3 and 5 relative to compounds 2, 4, and 6 (Table I) is consistent with differences between substituents on the C-3′ and C-5′ positions produced by the presence of a single methoxyl at C-3′ for petunidin.

Compounds 4 and 6. An identification of the aglycone produced by hydrolysis of peaks 4 and 6 as being the anthocyanidin malvidin (3,5,7,4′-tetrahydroxy-3′,5′-dimethoxyflavylium) (Table II) was supported by (a) MS data indicating a mol wt of 331; (b) ^{1}H-NMR resonances (Table I) matching authentic malvidin; (c) absence of a bathochromic shift in the presence of aluminum ion (Table I), which is consistent with the absence of *o*-dihydroxyl groups in the B ring; (d) UV_{max} and vis_{max} absorbances (Table I) matching authentic malvidin; and (e) co-chromatography with authentic malvidin.

Flavonols (Compounds 7–11)

Analyses of compounds purified from peaks 7 through 11 produced data consistent with published characteristics of flavonol glycosides (unhydrolyzed sample) and flavonol aglycones (hydrolyzed fraction) for UV/visible absorbances (24), ^{1}H-NMR spectra (24), and MS molecular ions (20, 23) (Table II, Fig. 2B). An *O*-glycoside linkage was inferred for these compounds because a 40-min acid hydrolysis released a flavonol aglycone from each intact compound. The aglycones shared several properties (Table III): (a) degeneration of the band I absorbance spectrum in the presence of CH_3ONa,

Table II. *Structures and Quantities of* nod *Gene-Inducing Flavonoids in Peaks 2 through 11 in Figure 1*

R_1, R_2, and R_3 are substituents to structures in Figure 2. Rates of release were calculated during the first 6 h of imbibition.

Peak	Substituent			Aglycone	Amount Released
	R_1	R_2	R_3		
					nmol seed^{-1} h^{-1}
	Anthocyanidins (Fig. 2A)				
2	-*O*-glycoside	OH	OH	Delphinidin	48
3	-*O*-glycoside	OMe[a]	OH	Petunidin	74
4	-*O*-glycoside	OMe	OMe	Malvidin	100
5	-*O*-glycoside	OMe	OH	Petunidin	72
6	-*O*-glycoside	OMe	OMe	Malvidin	129
	Flavonols (Fig. 2B)				
7	-*O*-glycoside	OH	OH	Myricetin	28
8	-*O*-glycoside	OH	H	Quercetin	14
9	-*O*-glycoside	OH	H	Quercetin	12
10	-*O*-glycoside	H	H	Kaempferol	10
11	-*O*-glycoside	H	H	Kaempferol	10

[a] OMe, methoxyl.

Table III. *UV/Visible Absorbance Maxima (nm) for Compounds from Peaks 7 through 11 in Figure 1*
Standard analytical reagents and procedures were used (24).

Peak[a]	Methanol	Reagent				
		CH_3ONa	$AlCl_3$	$AlCl_3/HCl$	CH_3COONa	CH_3COONa/H_3BO_3
7A	254, 267sh[b], 300sh, 376	263sh, 285sh, 317, 433dc[c]	269, 315sh, 352sh, 452	266, 273sh, 309sh, 366sh, 428	268, 333, 406dc	257, 301sh, 340sh, 393 II[d]
7G	255, 267sh, 300sh, 362	261sh, 280sh, 323, 402	271, 308sh, 363sh, 425	271, 306sh, 363sh, 405	271, 329, 396	256, 303sh, 382 I
8A and 9A	255, 269sh, 299sh, 372	243sh, 288sh, 331, 417dc	271, 301sh, 335, 458	266, 300sh, 362, 424	255sh, 274, 321, 395dc	260, 302sh, 387 II
8G	256, 267sh, 299sh, 358	272, 323sh, 409	274, 305sh, 365sh, 429	268, 302sh, 361, 407	272, 330sh, 388	261, 304sh, 380 I
9G	254, 264sh, 299sh, 356	270, 322sh, 409	273, 315sh, 366sh, 423	270, 304sh, 360, 409	273, 320sh, 390	261, 303sh, 379 I
10A and 11A	255sh, 267, 295sh, 325sh, 366	280, 319sh, 417dc	257sh, 269, 304sh, 350, 424	254sh, 269, 304sh, 349, 424	274, 305sh, 386dc	266, 296sh, 372 II
10G	265, 295sh, 349	274, 325, 399	274, 301sh, 344, 393	274, 301sh, 344, 393	274, 305sh, 385	266, 296sh, 352 I
11G	265, 295sh, 350	274, 327, 401	273, 302sh, 346, 388	274, 302sh, 352, 403	274, 303sh, 375	266, 292sh, 350 I

[a] A, aglycone; G, glycoside. [b] sh, spectral shoulder. [c] dc, decreasing intensity with time. [d] Procedure I or II (24).

which indicates free hydroxyl groups at C-3 and C-4′; (b) shifts or decreases in band II after adding CH_3COONa, which indicates a free hydroxyl at C-7; (c) four major absorption peaks in the presence of $AlCl_3$ and a shift after adding HCl (52–58 nm in band I, relative to the methanol spectrum), which indicates the presence of a free hydroxyl at C-5. Glycosylation at the C-3 position for peaks 7 through 11 was supported by the fact that hydrolysis produced bathochromic shifts in band I in methanol (14–17 nm) and in methanol with CH_3ONa (40–53 nm) (24). The presence of *o*-dihydroxyl groups on aglycones from compounds 7 through 9, but not 10 and 11, was supported by 15 to 17 nm shifts in band I in CH_3COONa/H_3BO_3.

Compound 7. An identification of the aglycone produced by hydrolysis of peak 7 as being the flavonol myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone) (Table II) was supported by (a) MS data indicating a mol wt of 318; (b) ^{1}H-NMR resonances matching authentic myricetin in [U-^{2}H]methanol: δ_H[3] ppm, 7.36 (2H, *s*, C-2′, C-6′), 6.36 (1H, *d*, J = 1.8 Hz, C-8), 6.16 (1H, *d*, J = 1.8 Hz, C-6); (c) UV/visible spectra in the presence and absence of shift reagents (Table III) matching authentic myricetin and consistent with published values (24); and (d) co-chromatography with authentic myricetin.

Compounds 8 and 9. During purification of peak 9, a smaller fraction with *nod*-inducing activity, representing about 15% of the original peak 9, was separated and subsequently termed compound 9. An identification of the aglycone produced by hydrolysis of peak 8 and compound 9 as being the flavonol quercetin (3,5,7,3′,4′-pentahydroxyflavone) (Table II) was supported by (a) MS data indicating a mol wt of 302; (b) ^{1}H-NMR resonances matching authentic quercetin in [U-^{2}H]methanol: δ_H ppm, 7.72 (1H, *d*, J = 1.8 Hz, C-6′), 7.62 (1H, *d*, J = 10.3 Hz, C-2′), 6.86 (1H, *d*, J = 4.3 Hz, C-5′), 6.38 (1H, *d*, J = 1.8 Hz, C-8), 6.16 (1H, *d*, J = 2.4 Hz, C-6); (c) UV/visible spectra in the presence and absence of shift reagents (Table III) matching authentic quercetin and consistent with published values (24); and (d) co-chromatography with authentic quercetin.

Compounds 10 and 11. An identification of the aglycone produced by hydrolysis of peaks 10 and 11 as being the flavonol kaempferol (3, 5, 7, 4′-tetrahydroxyflavone) (Table II) was supported by (a) MS data indicating a mol wt of 286; (b) ^{1}H-NMR resonances matching authentic kaempferol in [U-^{2}H]methanol: δ_H ppm, 8.08 (2H, *d*, J = 8.5 Hz, C-2′, C-6′), 6.89 (2H, *d*, J = 9.15 Hz, C-3′, C-5′); 6.38 (1H, *d*, J = 1.8 Hz, C-8); 6.16 (1H, *d*, J = 1.8 Hz, C-6); (c) UV/visible spectra in the presence and absence of shift reagents (Table III) matching authentic kaempferol and consistent with published values (24); and (d) co-chromatography with authentic kaempferol.

β-Galactosidase Induction Activity

Comparisons between authentic standards and putative aglycone compounds showed similar patterns of β-galactosidase induction over the concentration range of 10 nM to 10 μM with strain RBL1283 (Table IV) and the two *nodC-lacZ* containing strains used in this study (data not shown). Mean response curves (Fig. 3) comparing anthocyanidin and flavonol aglycones (Fig. 3B) showed that the former were more active inducers in the sense that the I_{50} was significantly lower ($P \leq 0.05$). Mean I_{50} for the three anthocyanidins was 360 nM, whereas that for the three flavonols was 980 nM. Comparing the same parameter for the anthocyanins and flavonol glycosides shows a similar trend, but the difference was not as great (Table IV). Measurable *nod*-inducing activity was detected for all glycosides at about 20% of the concentration required for aglycones (Fig. 3A). Maximum β-galactosidase

Table IV. *The* nod *Gene Inducing Activity of Natural Flavonoids Released from Bean Seeds and Authentic Standards*

Assays measured β-galactosidase activity produced from a *nodA-lacZ* fusion under the control of *nodD* from *R. leguminosarum* bv *phaseoli* in strain RBL1283. Compounds were assayed over a concentration range of 10 nM to 10 μM. Aglycones were produced by acid hydrolysis of naturally occurring glycosides.

Compound	Maximum Activity		I_{50}	
	Glycoside	Aglycone	Glycoside	Aglycone
	units β-galactosidase		*nM*	
Anthocyanidins				
2	1220	1050	230	581
Delphinidin		900		720
3	1210	1110	329	347
5	981	1030	250	315
Petunidin		1090		402
4	1120	1230	95	130
6	1450	1120	130	187
Malvidin		1440		290
Flavonols				
7	1210	821	780	1620
Myricetin		803		1400
8	980	911	128	649
9	940	813	97	418
Quercetin		900		674
10	1430	1020	362	726
11	1530	1420	374	839
Kaempferol		1230		625

activities were within the same range for the aglycones and their unhydrolyzed conjugates (Table IV). However, the I_{50} value of each compound increased following hydrolysis. Thus, an overall mean I_{50} value of 280 nM for the 10 glycosides increased to 580 nM for the aglycones (Fig. 3A). Comparisons of I_{50} values within the anthocyanidins showed a distinct trend indicating that increasing methoxylation of the B ring enhanced *nod*-inducing activity. Thus, delphinidin was less active than petunidin, which was less active than malvidin (Table IV).

Amounts of Compounds

On average, 86% of malvidin-3-*O*-monoglucoside and 92% of quercetin-3-*O*-monogalactoside added to imbibing seeds was recovered in the purification procedure. Using those values as standard correction factors for anthocyanidins (compounds 2–6) and flavonols (compounds 7–11), respectively, individual *nod*-inducing flavonoids were released at rates ranging from 10 to 129 nmol $seed^{-1}$ h^{-1} during the first 6 h of imbibition (Table II). Relatively more anthocyanin than flavonol glycoside was released: 423 *versus* 74 nmol $seed^{-1}$ h^{-1}.

DISCUSSION

Data in this report offer the first evidence that anthocyanins from legumes function naturally as *nod*-gene inducers in *Rhizobium* bacteria. Based on relative amounts released (Table II) and I_{50} values (Table IV), anthocyanins contributed nearly 10-fold more total *nod*-inducing activity than flavonol glycosides. Acid hydrolysis in conjunction with HPLC analyses showed that the six flavonoid aglycones identified as natural *nod*-gene inducers in this study (three anthocyanidins and three flavonols) were released from seeds as glycosides. Because there is no evidence that sugars alone induce *nod* genes in any rhizobia, this study focused on identifying the structures of the aglycones that are responsible for the *nod*-gene inducing activity.

The *nod* genes in *R. leguminosarum* bv *phaseoli* can be induced by various flavonoid structures. A recent report established that commercial samples of selected flavones, flavonols, flavanones, and isoflavones were active inducers in this bacterium (4). Data collected in this study show that PI165426CS bean seeds exploit some of that potential bacterial response by releasing 10 separate *nod*-gene-inducing flavonoid glycosides containing anthocyanidin (delphinidin, petunidin, and malvidin) and flavonol (myricetin, quercetin, and kaempferol) aglycones. All of these compounds, in addition to sharing somewhat similar flavonoid ring structures, have free hydroxyl groups at the C-4′, C-5, and C-7 positions (Fig. 2). The presence of so many structurally different *nod*-gene-inducing flavonoids in the seed rinse differs markedly from exudates of other legumes that have been studied in the absence of rhizobial symbionts (8, 13, 18, 29, 31, 32).

The 3-*O*-glycosylation present in all *nod*-gene-inducing flavonoids released from bean seeds (Table II) separates these compounds from natural inducers reported previously. Because anthocyanidins normally occur in glycosylated forms

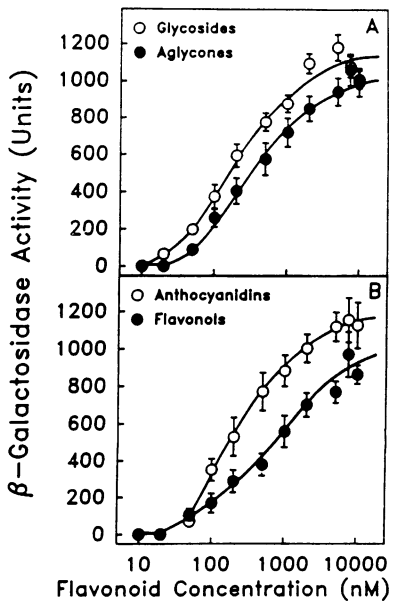

Figure 3. The *nod*-inducing activity of flavonoids released by a black-seeded bean line during imbibition. Data from separate anthocyanidin compounds identified from peaks 2 through 6 and flavonols from peaks 7 through 11 (Fig. 1A) were averaged from separate assays for β-galactosidase activity produced from a *nodA-lacZ* fusion under the control of *nodD* from *R. leguminosarum* bv *phaseoli* in rhizobial strain RBL1283. A, Assays of intact flavonoid compounds (*i.e.* anthocyanins and flavonol glycosides). B, Assays of hydrolyzed aglycones.

as anthocyanins (3, 12), their presence in that form is not surprising. After hydrolysis, the bean compounds remain active *nod*-gene inducers in *R. leguminosarum* bv *phaseoli* with the exposed C-3 hydroxyl group (Table IV), unlike results reported for tests with C-3 hydroxylated flavonoids in *R. leguminosarum* bv *trifolii, R. leguminosarum* bv *viceae, R. meliloti, R. fredii,* and *B. japonicum* (6, 8, 18, 30, 40). Only the wide-host-range *Rhizobium* NGR234 reportedly induces *nod* genes in response to flavonoids with C-3 hydroxylation (2).

Concentrations of flavonoid glycosides in this study were calculated from extinction coefficients reported for known glycosides containing the same aglycone. The validity of these calculations cannot be verified until accurate mol wts and crystalline amounts of the compounds in peaks 2 through 11 are available, but the nature of the sugar conjugate normally has little effect on the extinction coefficient of anthocyanins (10, 12) or flavonol glycosides (24, 38). Because each aglycone alone was an active *nod*-gene inducer, the 100% increase in I_{50} values calculated for aglycones *versus* glycosides (Fig. 3A) possibly reflects an increased uptake of the glycoside by bacteria in the assays.

Glycosylation of bean seed *nod*-gene inducers may have implications for both chemical and biological processes in the natural system. First, glycosylation increases the stability of anthocyanidins in light and alkaline conditions (3, 12). Second, flavonoid glycosides are more soluble in water, and consequently their movement in the soil solution may be facilitated. Whether that fact promotes nodulation by allowing flavonoid glycosides from bean seeds to signal to rhizobia over a greater distance or decreases nodulation in some cases by facilitating their percolation away from the seedling is unknown. *R. leguminosarum* bv *phaseoli* shows a positive chemotactic response to some flavonoids (1), but none identified in this study was tested. Due to these potential biological implications, the sugars in compounds 2 through 11 are being identified, and a complete analysis of the extremely soluble compound 1 is being done. Although many of the compounds contain several sugar residues (M. Hungria, unpublished data), there is no indication yet that the anthocyanins are as complex as some reported recently (17).

It is unclear how beans benefit from releasing so many different *nod*-gene inducers. Bean seed coat colors often result from mixtures of flavonols and anthocyanins (7, 21), and those compounds may have been selected for effects on plant predators or pathogens. In addition, anthocyanins and flavonols may help determine host legume specificity by inhibiting *nod*-gene expression in *R. legumoinosarum* bv *trifolii, R. leguminosarum* bv *viceae, B. japonicum,* and *R. meliloti,* in which induction is decreased by flavonoids with C-3 hydroxyls (6, 8, 19, 30). There is even a claim that myricetin has antibiotic effects on *R. leguminosarum* bv *trifolii* (9). As we develop a more comprehensive understanding of flavonoids that are actually released from seeds and assess the biological effects of those compounds on microbes, it may be possible to use the diversity of seed coat flavonoids to enhance rhizobial nodulation and to promote favorable interactions with other soil microbes.

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LITERATURE CITED

1. **Aguilar JMM, Ashby AM, Richards AJM, Loake GJ, Watson MD, Shaw CH** (1988) Chemotaxis of *Rhizobium leguminosarum* biovar *phaseoli* towards flavonoid inducers of the symbiotic nodulation genes. J Gen Microbiol **134:** 2741–2746
2. **Bassam BJ, Djordjevic MA, Redmond JW, Batley M, Rolfe BG** (1988) Identification of a *nodD*-dependent locus in the *Rhizobium* strain NGR234 activated by phenolic factors secreted by soybeans and other legumes. Mol Plant-Microbe Interact **1:** 161–168
3. **Brouillard R** (1988) Flavonoids and flower colour. *In* JB Harborne, ed, The Flavonoids, Advances in Research Since 1980. Chapman and Hall, London, pp 525–538
4. **Davis EO, Johnston AWB** (1990) Regulatory function of the three *nodD* genes of *Rhizobium leguminosarum* biovar *phaseoli.* Mol Microbiol **4:** 933–941
5. **Dickson MH, Petzoldt R** (1988) Deleterious effects of white seed due to *p* gene in beans. J Am Soc Hortic Sci **113:** 111–114
6. **Djordjevic MA, Redmond JW, Batley M, Rolfe BG** (1987) Clover secrete specific phenolic compounds which either stimulate or repress *nod* gene expression in *Rhizobium trifolii.* EMBO J **6:** 1173–1179
7. **Feenstra WJ** (1960) Biochemical aspects of seedcoat colour inheritance in *Phaseolus vulgaris* L. Meded Landbouwhogeschool **60:** 1–53
8. **Firmin JL, Wilson KE, Rossen L, Johnston AWB** (1986) Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. Nature **324:** 90–92
9. **Fottrell PF, O'Connor S, Masterson CL** (1964) Identification of the flavonol myricetin in legume seeds and its toxicity to nodule bacteria. Ir J Agric Res **3:** 246–249
10. **Francis FJ** (1982) Analysis of anthocyanins. *In* P Markakis, ed, Anthocyanins as Food Colors. Academic Press, New York, pp 181–207
11. **Fuleki T, Francis FJ** (1968) Quantitative methods for anthocyanins. 1. Extraction and determination of total anthocyanin in cranberries. J Food Sci **33:** 72–77
12. **Harborne JB** (1967) Comparative Biochemistry of the Flavonoids. Academic Press, New York
13. **Hartwig UA, Maxwell CA, Joseph CM, Phillips DA** (1990) Chrysoeriol and luteolin released from alfalfa seeds induce *nod* genes in *Rhizobium meliloti.* Plant Physiol **92:** 116–122
14. **Jain V, Garg N, Nainawatee HS** (1990) Naringenin enhanced efficiency of *Rhizobium meliloti*-alfalfa symbiosis. World J Microbiol Biotech **6:** 434–436
15. **Kagan J** (1967) Myricetin 3-β-galactosidase, the main flavonoid from *Oenothera lavandulaefolia,* T. and G. Phytochemistry **6:** 317–318
16. **Kapulnik Y, Joseph CM, Phillips DA** (1987) Flavone limitations to root nodulation and symbiotic nitrogen fixation in alfalfa. Plant Physiol **84:** 1193–1196
17. **Kondo T, Ueda M, Goto T** (1990) Structure of ternatin B1, a pentaacylated anthocyanin substituted on the B-ring asymmetrically with two long chains. Tetrahedron **46:** 4749–4756
18. **Kosslak RM, Bookland R, Barkei J, Paaren HE, Appelbaum ER** (1987) Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavone isolated from *Glycine max.* Proc Natl Acad Sci USA **84:** 7428–7432
19. **Kosslak RM, Joshi RS, Bowen BA, Paaren HE, Appelbaum ER** (1990) Strain-specific inhibition of *nod* gene induction in *Bradyrhizobium japonicum* by flavonoid compounds. Appl Environ Microbiol **56:** 1333–1341
20. **Koster CG, Heerma W, Dijkstra G, Niemann GJ** (1985) Fast atom bombardment of flavonols. Biomed Mass Spectrom **12:** 596–601

21. **Leakey CLA** (1988) Genotypic and phenotypic markers in common bean. *In* P Gepts, ed, Genetic Resources of *Phaseolus* Beans. Kluwer Academic, Dordrecht, pp 245–327
22. **Long SR** (1989) Rhizobium-legume nodulation: life together in the underground. Cell **56:** 203–214
23. **Mabry TJ, Markham KR** (1975) Mass spectrometry of flavonoids. *In* JB Harborne, TJ Mabry, H Mabry, eds, The Flavonoids. Academic Press, New York, pp 78–126
24. **Mabry TJ, Markham KR, Thomas MB** (1970) The Systematic Identification of Flavonoids. Springer-Verlag, New York
25. **Markham KR, Mabry TJ** (1975) Ultraviolet-visible and proton magnetic resonance spectroscopy of flavonoids. *In* JB Harborne, TJ Mabry, H Mabry, eds, The Flavonoids, Vol 1. Academic Press, New York, pp 45–77
26. **Maxwell CA, Hartwig UA, Joseph CM, Phillips DA** (1989) A chalcone and two related flavonoids released from alfalfa roots induce *nod* genes of *Rhizobium meliloti*. Plant Physiol **91:** 842–847
27. **Miller JH** (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 352–355
28. **Neves MCP, Hungria M** (1987) The physiology of nitrogen fixation in tropical grain legumes. CRC Crit Rev Plant Sci **6:** 267–321
29. **Peters NK, Frost JW, Long SR** (1986) A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science **233:** 977–980
30. **Peters NK, Long SR** (1988) Alfalfa root exudates and compounds which promote or inhibit induction of *Rhizobium meliloti* nodulation genes. Plant Physiol **88:** 396–400
31. **Recourt K, Schripsema J, Kijne JW, Van Brussel AAN, Lugtenberg BJJ** (1991) Inoculation of *Vicia sativa* subsp. *nigra* roots with *Rhizobium leguminosarum* biovar *viciae* results in release of *nod* gene activating flavanones and chalcones. Plant Mol Biol **16:** 841–852
32. **Redmond JW, Batley M, Djordjevic MA, Innes RW, Kuempel PL, Rolfe BG** (1986) Flavones induce expression of nodulation genes in *Rhizobium*. Nature **323:** 632–635
33. **Sadowsky MJ, Olson ER, Foster VE, Kosslak RM, Verma DPS** (1988) Two host inducible genes in *Rhizobium fredii* and characterization of the inducible compound. J Bacteriol **170:** 171–178
34. **Spaeth EC, Rosenblatt DH** (1950) Partition chromatography of synthetic anthocyanidin mixtures. Anal Chem **22:** 1321–1326
35. **Spaink HP, Okker RJH, Wijffelman CA, Pees E, Lugtenberg BJJ** (1987) Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol Biol **9:** 27–39
36. **Strack D, Wray V** (1989) Anthocyanins. *In* PM Dey, JB Harborne, eds, Plant Phenolics. Academic Press, London, pp 325–326
37. **Vincent JM** (1970) A Manual for the Practical Study of the Root-Nodule Bacteria. Blackwell Scientific, Oxford
38. **Weast RC,** ed (1969) Handbook of Chemistry and Physics. The Chemical Rubber Co, Cleveland
39. **Yokotsuka K, Nishino N** (1990) Extraction of anthocyanins from Muscat Bailey A grape skins. J Ferment Bioeng **69:** 328–334
40. **Zaat AJ, Schripsema J, Wijffelman CA, Van Brussel AAN, Lugtemberg BJJ** (1989) Analysis of the major inducers of the *Rhizobium nodA* promoter from *Vicia sativa* root exudate and their activity with different *nodD* genes. Plant Mol Biol **13:** 175–188