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Strain-Specific Inhibition of *nod* Gene Induction in *Bradyrhizobium japonicum* by Flavonoid Compounds

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A broad-host-range plasmid, pEA2-21, containing a *Bradyrhizobium japonicum nodABC'-'lacZ* translational fusion was used to identify strain-specific inhibitors of the genes required for soybean nodulation, the common *nod* genes. The responses of type strains of *B. japonicum* serogroups USDA 110, USDA 123, USDA 127, USDA 129, USDA 122, and USDA 138 to *nod* gene inhibitors were compared. Few compounds inhibited *nod* gene expression in *B. japonicum* USDA 110. In contrast, *nod* gene expression in strains belonging to several other serogroups was inhibited by most of the flavonoids tested. However, the application of two of these strain-specific compounds, chrysin and naringenin, had little effect on the pattern of competition between indigenous and inoculum strains of *B. japonicum* in greenhouse and field trials. Preliminary studies with radiolabeled chrysin and naringenin suggest that the different responses to *nod* gene inhibitors may be partly due to the degree to which plant flavonoids can be metabolized by each strain.

The symbiotic relationship between *Bradyrhizobium japonicum* and its soybean host is of considerable agronomic importance. In many soils of the midwestern United States, inoculation of soybeans with effective strains of *B. japonicum* often fails to increase yields (7, 14). This is partly due to the failure of inoculant strains (e.g., USDA 110) to displace highly competitive, but often less efficient, indigenous strains (e.g., serogroup 123) from the nodules (14, 16). Pre-exposure (21) and split-root (19) experiments have indicated that the strain which initiates the first nodules has a competitive advantage over those which follow. These experiments did not establish whether it was preinfection or postinfection events which were critical in determining the outcome of competition between strains for nodulation of their soybean host. The competitive ability of a given strain is also influenced by genetic background (25, 31, 33, 38), soybean genotype (8, 17), and abiotic and biotic factors (19, 20, 34).

In *Bradyrhizobium* and *Rhizobium* species, several of the genes required for the early stages of nodule formation, including the common *nod* genes, are regulated at the level of transcription by flavonoid compounds present in legume roots and exudates (for a review, see reference 23). Differences among strains in their response to particular compounds have been shown to affect host range for nodulation (23). It is possible that differences in *nod* gene induction or inhibition or both among strains with the same host range might also be a factor in competition for nodule formation in soils containing indigenous populations of *Bradyrhizobium* or *Rhizobium* species. In the *B. japonicum*-soybean association, the isoflavones daidzein and genistein have been identified as the natural inducers of the common *nod* genes (3, 22). In this study, a plasmid carrying a *nodC'-'lacZ* translational fusion (22) was used to identify inhibitors of *nod* gene induction in *B. japonicum* serogroups 123, 127, 129, 110, 138, and 122. Compounds were found which in some or all of the strains tested inhibited *nod* gene expression. Strain-specific inhibitors of *nod* gene induction were used in greenhouse and field trials to determine their effects on nodule occupancy by inoculum and indigenous *B. japonicum* strains.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. japonicum* USDA 110, USDA 123, USDA 127, USDA 129, USDA 122, and USDA 138 were obtained from M. Sadowsky, Beltsville, Md. *Pseudomonas fluorescens* and *P. cepacia* J82-R were obtained from T. McLoughlin, Stine Microbial Products, Madison, Wis., and maintained on nutrient broth (Difco laboratories, Detroit, Mich.). The construction of pEA2-21 has been described in detail elsewhere (22); this plasmid contains the *nodD nodABC'* region from *B. japonicum* USDA 123 cloned into a broad-host range vector. A translational fusion was created between the *nodC* gene and the *lacZ* gene during the construction of pEA2-21. *B. japonicum* USDA 110, USDA 123, and USDA 138 containing pEA2-21 were obtained as described previously (22). pEA2-21 was introduced into *B. japonicum* USDA 122, USDA 127, and USDA 129 by electroporation (10; B. A. Bowen, manuscript in preparation). Bacteria were grown in A1EHM (31) medium to an A_{600} of 1.0, washed three times in high-pressure liquid chromatography-grade water, and suspended at a density of at least 10^{12} cells per ml. Cells were mixed with pEA2-21 (1 ng/μl) on ice and electroporated at an initial voltage gradient of 17.5 kV/cm. A total energy of 0.55 J was delivered. Transformants were selected on A1EHM medium containing 75 μg of tetracycline per ml (22).

Source of flavonoid compounds. Genistein, kaempferol, and luteolin were obtained from ICN, Cleveland, Ohio; quercetin, morin, umbelliferone, and chrysin were from Sigma Chemical Co., St. Louis, Mo.; prunetin, daidzein, 5-hydroxyflavone, 7-hydroxyflavone, eriodictyol, galangin,

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and formononetin were from Spectrum Chemical, Gardena, Calif.; naringenin, apigenin, and biochanin A were from Aldrich Chemical Co., Milwaukee, Wis.; and coumestrol was from Eastman Kodak Co., Rochester, N.Y. 4',7-Dihydroxyflavone was synthesized by published procedures (12).

β-Galactosidase assays. Compounds were tested for inducing or inhibitory activities by using liquid-grown cultures. *B. japonicum* strains containing pEA2-21 were grown with shaking to an A_{600} of 0.200 in A1EHM medium at 28°C. Compounds were added to 2 ml of culture by dilution from ethanol stocks. Compounds tested for inhibitory activities were added at the same time as the natural inducer, daidzein. The concentration of daidzein (5 μM for USDA 138, 1 μM for all other strains) used was based on a concentration curve constructed for each strain. The level used was sufficient to induce approximately 75% of maximal β-galactosidase activity for each strain. Cells were induced for 21 h, permeabilized with chloroform containing 0.1% sodium dodecyl sulfate, and assayed as described by Miller (26). Three replicate cultures were assayed for each compound or pair of compounds tested on two occasions. Reported values are the means of three replicate samples from one representative experiment.

Radiolabeling experiments. [^{3}H]chrysin or [^{3}H]naringenin were synthesized as follows: 970 mg (9.5 mmol) of acetic anhydride plus 180 μl (10 mmol) of ^3H_2O (90 Ci/mmol) were heated in a sealed reaction vial at 55°C for 10 h. The reaction was cooled on ice, and the [^{3}H]acetic acid (approximately 45 mCi/mmol) was transferred to a 2.0-ml glass ampoule containing 2 mg of platinum(IV) oxide and 4 mg of either chrysin or naringenin. The mixture was frozen on dry ice, and the ampoule was sealed under argon gas. The sealed ampoule was heated at 115°C for 20 to 22 h, cooled to room temperature, and evaporated to dryness in a fume hood under argon gas. The crude material was taken up in 2 ml of chloroform-methanol (8:2) and filtered through a small amount of Celite prewet in the same solvent. The filtrate was loaded on a 250-μm silica thin-layer chromatography plate (20 by 20 cm) which was developed in chloroform-methanol (8:2). Approximately 1 to 2 mg of ^{3}H-labeled chrysin or naringenin with specific activity of ≥100 mCi/mmol was purified by eluting bands which comigrated with an authentic sample of either compound in chloroform-methanol (1:1).

B. japonicum strains were induced as described previously, except that 1 μM concentrations of the radiolabeled compounds were added to 9 μM concentrations of nonradiolabeled chrysin or naringenin to give a specific activity of 2×10^6 cpm/50 ml of culture. Overnight cultures of the *Pseudomonas* strains were used to inoculate fresh tubes of nutrient broth. Cultures were grown to early log phase, with shaking, at 28°C, and radiolabeled chrysin or naringenin was added. Bacteria were harvested 21 h after the addition of the compounds, spun down, washed, and suspended in scintillation cocktail. Culture supernatants were dried down and suspended in a small volume of water and scintillation cocktail. Radioactivity in each fraction was measured in counts per minute, using a liquid scintillation counter. High-pressure liquid chromatography (22) was used in conjunction with radiolabeled experiments to verify the absence or presence of chrysin and naringenin in culture supernatants.

Strain identification. The percentage of nodules occupied by each strain was determined by using an enzyme-linked immunosorbent assay (2, 18, 29). A minimum of 25% of the nodules from each replicate were identified. Polyclonal antibodies were prepared against each of the USDA type strains as described before (27, 31). Cross-reacting antibodies were removed by the procedure of Schmidt et al. (33).

Effect of inhibitors on nodule occupancy. A 25% soil mix containing Dane County (Wisconsin) top soil, perlite, Turface, peat, and rice hulls was used for the greenhouse trials. Five seeds of *Glycine max* L. Merrill cv. J103 (Jacques Seed Co., Prescott, Wis.) were sown in 6-in. (15.24-cm) pots, inoculated with mid-log-phase cultures, and watered with 200 ml of an aqueous solution of chrysin, naringenin, or water. Inoculum density was estimated from the A_{600} of cells grown in A1EHM medium, and dilutions were made in water to obtain approximately 10^6 to 10^7 cells per ml. The cell density of the inoculum strains was confirmed by plate counts on A1EHM agar medium. The serotype of the inoculum strains was verified prior to inoculation by using strain-specific fluorescent antibodies (20, 21). After germination, seedlings were thinned to two seeds per pot. Completely randomized designs were used to test the following parameters on nodule occupancy: (i) efficacy of inhibitors; (ii) concentration of inhibitors; and (iii) time at which the inhibitors were added. Plants were harvested 4 to 5 weeks after planting, and nodule occupancy was determined by enzyme-linked immunosorbent assay.

Field trials were used to test the efficacy of selected *nod* gene inhibitors and the method of inhibitor application on the pattern of competition between indigenous *B. japonicum* serogroups. Two test sites were examined over two growing seasons (1987 to 1988). The first site contained 10^3 bradyrhizobia per g of soil, as determined by the most-probable-number technique (39), and was a silt loam (Dane County, Wis.). The second site was a sandy loam (Columbia, County, Wis.) and contained <10 bradyrhizobia per g of soil. For both sites, completely randomized block designs were used. Inhibitors were applied as aqueous solutions or incorporated as a powder in peat. For liquid application, the inhibitors were applied 3, 4, and 5 days after planting (cv. J103) at a rate of 250 ml/ft. Four 100-ft (30.5-m) rows were used for each replicate, and each treatment was replicated three times. Plants were harvested 52 days after planting, and nodule occupancy was determined by enzyme-linked immunosorbent assay. A total of 344 nodules were serotyped for each replicate. For peat inoculation, chrysin or naringenin was incorporated as a powder at a rate of 100 μg per seed with *B. japonicum* USDA 110 (4×10^7 cells per seed) or USDA 138 (5×10^7 cells per seed). Strains were grown as described for the greenhouse trials. Seeds of cv. J103 were sown in 4-ft (121.9-cm) rows, with two rows used per replicate. Each treatment was replicated five times. Plants were harvested 47 days after planting. Eighty-six nodules were serotyped per replicate, using enzyme-linked immunosorbent assay.

The results from the greenhouse and field trials were analyzed by using two- and three-way analyses of variance (35). Differences between treatment means were analyzed by using Tukey's Honestly Significant Difference test (35). For statistical analysis, percent occupancy data was transformed by using the arc-sine transformation (35). Data shown in Tables 2 and 3 are presented as untransformed percentages.

RESULTS

Induction of the common *nod* genes in *B. japonicum* USDA 110, 123, 127, 129, 122, and 138 by daidzein and genistein is shown in Fig. 1. Although the expression levels of the nodulation genes varied considerably between strains, daidzein was the preferred inducer for all strains tested. In

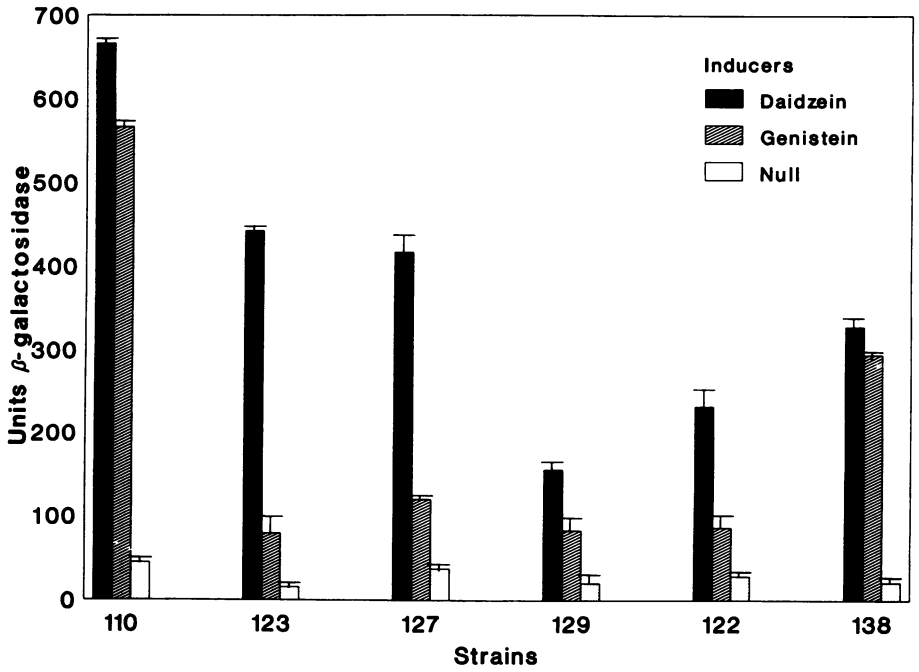


FIG. 1. Induction of the *nodABC'-'lacZ* genes by the isoflavones daidzein and genistein. *B. japonicum* USDA 110, 123, 127, 129, and 122 were tested with 1 μM daidzein or genistein. Strain USDA 138 was tested with 5 μM concentrations of the two compounds. The null treatment shows β-galactosidase activity in the absence of inducers. Values shown are the means ± standard deviations of three replicates.

USDA 110, 129, and 138, induction by genistein was similar to that observed with daidzein. In USDA 123, 127, and 122, expression of the nodulation genes by genistein was reduced approximately 50% when compared with the levels obtained with daidzein.

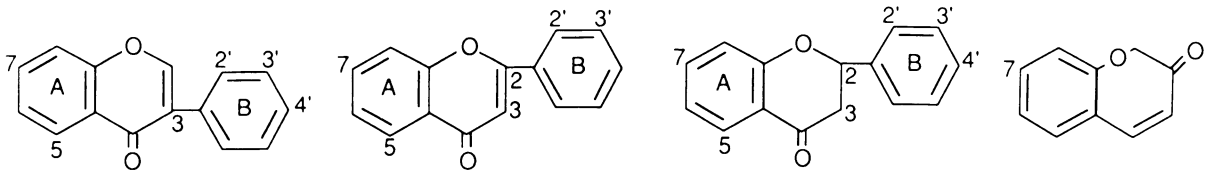
Flavonoid compounds similar in structure to daidzein and genistein (Table 1) were tested for their ability to inhibit *nod* gene induction by daidzein in *B. japonicum* USDA 110, USDA 123, USDA 127, USDA 129, USDA 122, and USDA 138 (Fig. 2). All 17 compounds inhibited induction to some extent in one or more of the strains. Three compounds (formononetin, kaempferol, and galangin) inhibited all six strains. Two compounds (prunetin and 5-hydroxyflavone) inhibited USDA 127, but had little effect on any of the other strains. Biochanin A was unique in that it inhibited USDA 110 and USDA 127, but no other strain. Eleven other compounds had no effect on induction in USDA 110, but inhibited most or all of the other strains to varying extents. The flavones chrysin, luteolin, apigenin, 4',7-dihydroxyflavone, and 7-hydroxyflavone and the flavanone naringenin were particularly potent inhibitors of strains other than USDA 110 (Fig. 2). Inhibitory activities of eriodictyol, quercetin, and morin were highly variable between the strains. Umbelliferone and coumestrol were generally weak inhibitors of *nod* gene expression in *B. japonicum*. Similar results were obtained when either genistein (all strains) or root exudate (only USDA 110 and USDA 123 were tested) was substituted for daidzein (data not shown).

An example of strain-specific inhibition of *nod* gene induction is shown in Fig. 3. *nod* gene induction in USDA 123 was reduced approximately 50% by a 1:1 ratio of chrysin/daidzein. When the chrysin/daidzein ratio was increased from 6:1 to 50:1, *nod* gene expression in USDA 123 was reduced 5- to 13-fold, respectively. *nod* gene expression in USDA 110 was unaffected by all ratios used. *nod* gene inhibition by chrysin in USDA 123 could be reversed by increasing the levels of daidzein and maintaining the concentration of chrysin at 1 μM (data not shown).

The fate of radiolabeled chrysin and naringenin was investigated to address why *nod* gene induction in USDA 110 was not affected by either compound (Fig. 4). The proportion of radiolabel remaining in the culture supernatant after 21 h was used to estimate the conversion of these compounds to volatile materials. Counts obtained from bacterial pellets were <1% of the total counts and are not shown. The percent volatile counts from USDA 110 cultures treated with either [^{3}H]chrysin or [^{3}H]naringenin were considerably higher than those in the uninoculated controls or in *B. japonicum* USDA 138 and USDA 127 and *P. fluorescens* (Fig. 4). The percent volatile counts obtained with *P. cepacia* J82-R was similar to those observed with USDA 110. Additional evidence for the degradation of chrysin and naringenin by USDA 110 was provided by the examination of culture supernatants by high-pressure liquid chromatography. No trace of chrysin or naringenin was detected in culture supernatants obtained from USDA 110. In contrast, both compounds were observed in culture supernatants obtained from USDA 123 (data not shown). These results suggest that chrysin and naringenin may fail to inhibit *nod* gene expression in USDA 110 because they are degraded more rapidly than in the other *B. japonicum* strains tested.

The effects of chrysin and naringenin on nodule occupancy by indigenous and inoculum strains of *B. japonicum* were tested in soils containing indigenous populations of *Bradyrhizobium* spp. because previous studies (20, 21) have shown that the pattern of competition between USDA 110 and USDA 123 is reversed in vermiculite and in *B. japonicum*-free soil. Results from one greenhouse trial are presented in Table 2. Nodule occupancy by members of serogroup 123 was significantly altered by the addition of 10 μM

TABLE 1. Chemical structures of compounds assayed for inducing and inhibitory activities



ISOFLAVONES	FLAVONES/FLAVONOLS			FLAVANONES		COUMARINS
COMPOUNDS	SUBSTITUTION PATTERN					
	3	5	7	2'	3'	4'
ISOFLAVONES						
Daidzein (DAI)			OH			OH
Genistein (GEN)		OH	OH			OH
Formononetin (FOR)			OH			OCH_3
Biochanin A (BIO)		OH	OH			OCH_3
Prunetin (PRU)		OH	OCH_3			OH
FLAVONES						
Chrysin (CHR)		OH	OH			
Luteolin (LUT)		OH	OH		OH	OH
Apigenin (API)		OH	OH			OH
4',7-Dihydroxy (DHF)			OH			OH
5-Hydroxy (5OH)		OH				
7-Hydroxy (7OH)			OH			
FLAVANONES/FLAVONOLS						
Naringenin (NAR)		OH	OH			OH
Eriodictyol (ERI)		OH	OH		OH	OH
Quercetin (QUE)	OH	OH	OH		OH	OH
Kaempferol (KAE)	OH	OH	OH			OH
Galangin (GAL)	OH	OH	OH			
Morin (MOR)	OH	OH	OH	OH		OH
COUMARINS						
Umbelliferone (UMB)			OH			
Coumestrol (COU)						

chrysin or 10 μM naringenin or by the use of USDA 110 as an inoculum strain. The application of chrysin or naringenin at various times after planting did not improve nodule occupancy by indigenous strains belonging to serogroup 110 or by the inoculum strain. In seven subsequent greenhouse trials, changes in nodule occupancy by members of serogroup 123 following the addition of chrysin or naringenin did not deviate significantly from the changes observed in the absence of these compounds (data not shown).

When chrysin and naringenin were used in field trials, neither compound affected nodule occupancy by the inoculum strains or by indigenous strains belonging to serogroups 110, 123, 122, and "others" (Table 3). However, nodule occupancy by strains belonging to serogroup 138 increased when naringenin was applied as a 50 μM aqueous solution. Incorporation of the two compounds as part of a peat inoculum had no observable effect on nodule occupancy by any of the strains serotyped (Table 3). The only factor which consistently altered the pattern of competition between indigenous *B. japonicum* serogroups was inoculation with either USDA 110 or USDA 138 (Table 3).

DISCUSSION

The most potent inhibitors of *nod* gene expression in *B. japonicum* were flavone, flavanone, and flavonol compounds which carried substitutions at the 5, 7, and/or 4' positions (Fig. 2 and Table 1). These are the same positions which are substituted in the natural inducers, the isoflavones daidzein and genistein (Table 1). Substitution of the flavone, flavanol, or flavanone skeleton at the 5 (e.g., 5-hydroxyflavone) position only or at the 2' (e.g., morin) or 3' (e.g., eriodicytol, quercetin) position resulted in weak inhibitory activities or none. Thus, compounds which share structural similarities with either daidzein or genistein (e.g., 7-hydroxyflavone, chrysin, naringenin, and kaempferol) were the most effective inhibitors of *nod* gene induction in *B. japonicum*. Potent

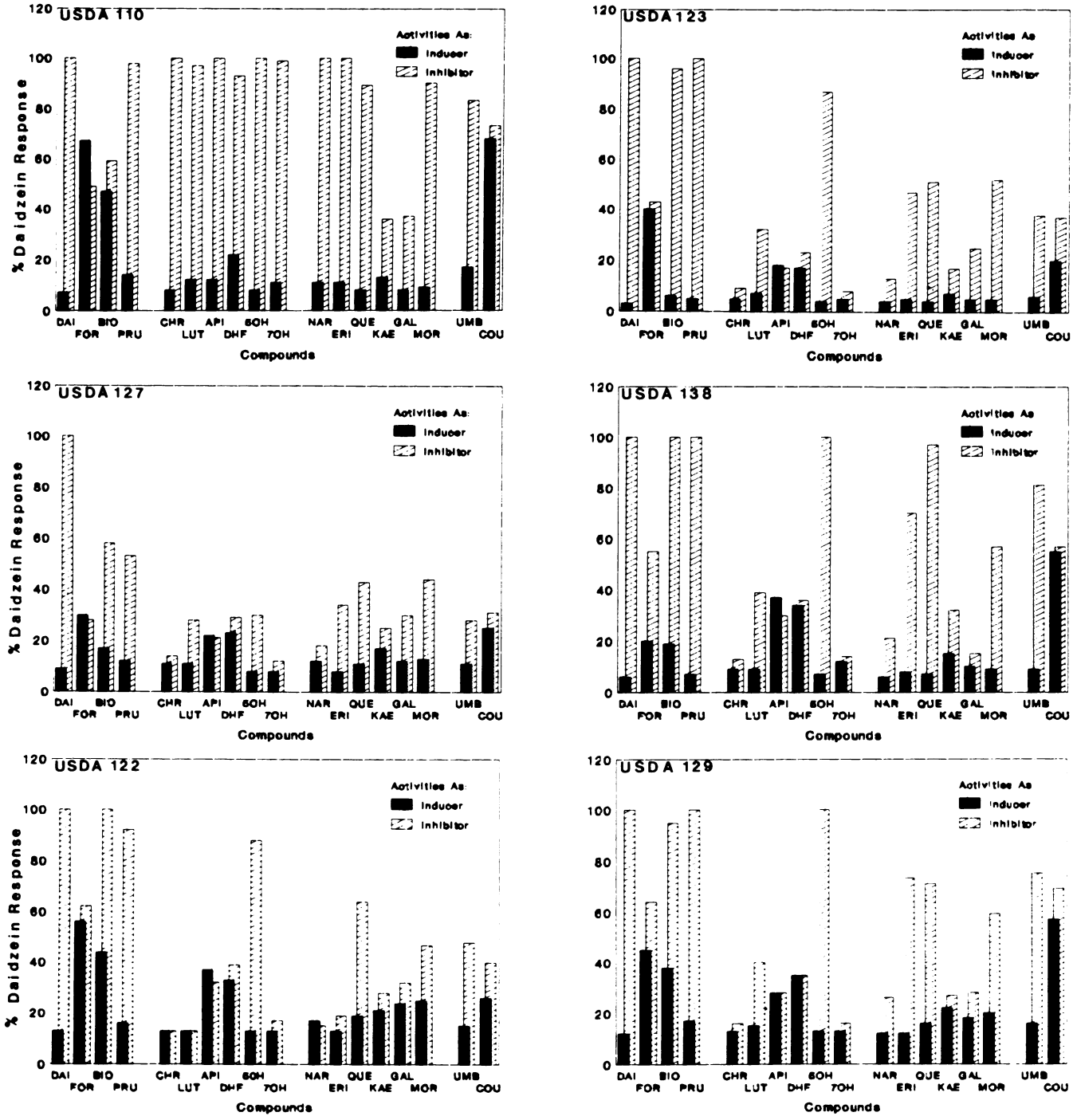


FIG. 2. Induction and inhibition profiles of *B. japonicum* strains in response to plant flavonoids. The response for each strain was normalized by converting units of β-galactosidase (mean of three replicates) to percentage of the response observed with daidzein alone. Induction of the *nodABC'-'lacZ* fusion to flavonoid compounds is represented by the black bars in each histogram. Induction experiments were carried out with 10 μM concentrations of all compounds, except daidzein (1 μM). Inhibition, in the presence of 1 μM daidzein plus a 10 μM concentration of each flavoniod compound, is represented by the striped bars. USDA 138 was tested with 5 μM daidzein plus a 10 μM concentration of each flavonoid compound. For each strain, the response to daidzein is shown as the first striped bar, and expression in the absence of flavonoid compounds is represented by the first black bar in each series. Compounds are abbreviated as in Table 1.

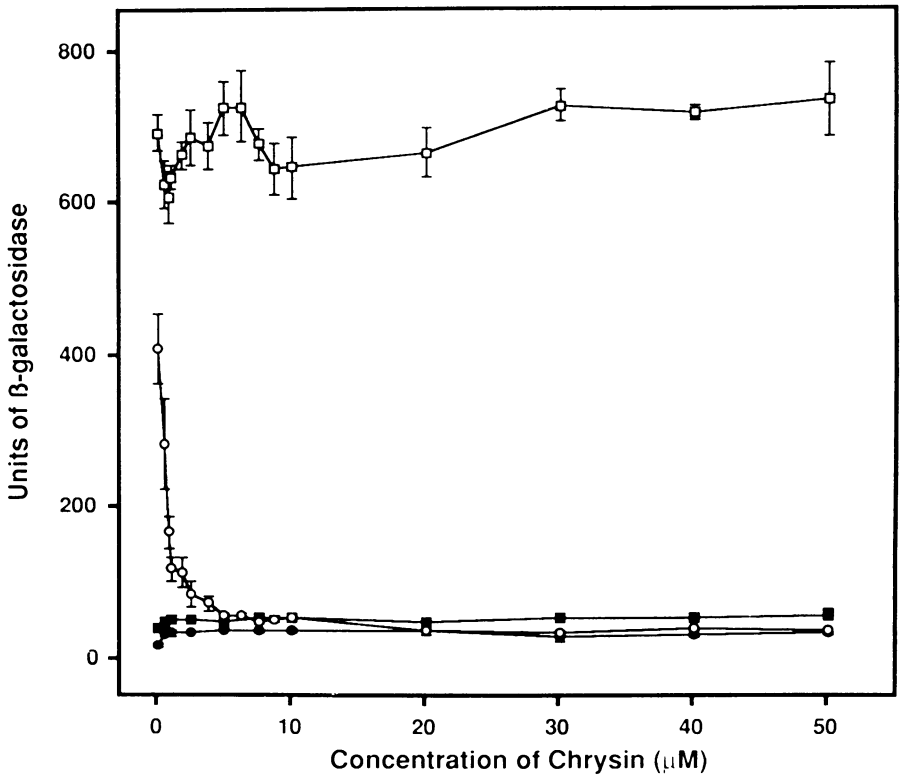


FIG. 3. Strain-specific inhibition of nodulation genes by chrysin. Values for β-galactosidase activity are given as the mean ± standard deviation for *B. japonicum* USDA 110(pEA2-21) and USDA 123(pEA2-21). The four curves depict *nodABC-lacZ* induction in the absence (■) or presence (□) of 1 μM daidzein for USDA 110 and *nodABC-lacZ* induction in the absence (●) or presence (○) of 1 μM daidzein for USDA 123. Controls consisted of cells grown in the absence of chrysin and daidzein (values represented at 0 μM; curves ● and ■) and in the presence of 1 μM daidzein (values represented at 0 μM; curves ○ and □).

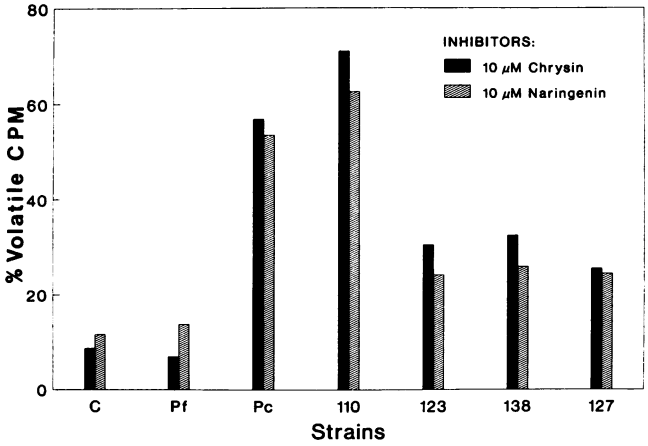


FIG. 4. Response of *B. japonicum*, *P. cepacia*, and *P. fluorescens* strains to radiolabeled [^{3}H]chrysin and [^{3}H]naringenin. Symbols on the *x* axis: C, no cell control (broth only); Pf, *P. fluorescens*; Pc, *P. cepacia*; 110, *B. japonicum* USDA 110; 123, *B. japonicum* USDA 123; 138, *B. japonicum* USDA 138; 127, *B. japonicum* USDA 127. Percentage of volatile counts (cpm) was determined as, 100 × (cpm T_0 − cpm T_{21})/cpm T_0 = % volatile counts. See Materials and Methods for further details.

inhibitors of *nod* gene induction in *Rhizobium leguminosarum* (11), *R. trifolii* (9), and *R. meliloti* (30) also exhibit strong structural similarities to the natural inducers in those systems. These finding suggest that inhibitors of *nod* genes may act competitively against inducers at a common target site, such as the *nodD* protein or an uptake system (9, 11, 32).

Few of the compounds tested inhibited *nod* gene expression in *B. japonicum* USDA 110. Preliminary studies with radiolabeled chrysin and naringenin (Fig. 4) and high-pressure liquid chromatography analysis (data not shown) suggest that these compounds may fail to inhibit *nod* gene induction in USDA 110 because they are more extensively degraded than in the other *B. japonicum* strains tested. Metabolic pathways for ring cleavage of flavonoids and similar phenolic compounds exist in other bacteria (40), including several *Pseudomonas* species (5, 42).

Preliminary results with chrysin and naringenin in greenhouse (Table 2) and in field trials (data not shown) indicated that either inhibitor was capable of altering the pattern of nodule occupancy among indigenous strains. However, subsequent greenhouse and field studies (Table 3; data not shown) indicated that the effects of chrysin and naringenin on the pattern of competition between indigenous *B. japonicum* strains were minimal and highly variable. There are several possible explanations for the failure of these compounds to alter the pattern of competition between strains of *B. japonicum* consistently. For example, the half-life of these compounds in soil may be highly

TABLE 2. Effect of inhibitor application on nodule occupancy in greenhouse-grown soybeans

Treatment[a]			Nodule occupancy (% of total)[b]				Ratio 123/110[c]
Inoculum	Compound	Application	USDA 110	USDA 123	USDA 110 + 123	Other	
None	None	T_0	3.41	70.12[d]	4.24[de]	22.23	
None	None	T_0, T_2	3.41	70.12[d]	4.24[de]	22.23	20.6
None	None	T_0, T_2, T_6	3.41	70.12[d]	4.24[de]	22.23	
None	Chrysin	T_0	4.31	45.86[efg]	2.43[fg]	47.40	
None	Chrysin	T_0, T_2	3.72	48.44[efg]	2.74[fg]	45.10	10.6
None	Chrysin	T_0, T_2, T_6	2.04	48.39[efg]	1.15[fg]	48.42	
None	Naringenin	T_0	3.49	51.65[ef]	0.65[efg]	44.22	
None	Naringenin	T_0, T_2	2.79	61.44[ef]	2.39[efg]	33.37	15.0
None	Naringenin	T_0, T_2, T_6	4.24	50.66[ef]	4.34[efg]	40.76	
USDA 110	None	T_0	12.86	54.03[ef]	8.51[de]	24.61	
USDA 110	None	T_0, T_2	12.86	54.03[ef]	8.51[de]	24.61	4.2
USDA 110	None	T_0, T_2, T_6	12.86	54.03[ef]	8.51[de]	24.61	
USDA 110	Chrysin	T_0	11.30	43.33[fg]	5.22[efg]	40.15	
USDA 110	Chrysin	T_0, T_2	15.76	45.22[fg]	6.97[efg]	32.05	3.6
USDA 110	Chrysin	T_0, T_2, T_6	9.97	41.24[fg]	4.03[efg]	44.76	
USDA 110	Naringenin	T_0	12.65	46.51[efg]	10.48[def]	30.36	
USDA 110	Naringenin	T_0, T_2	13.12	49.42[efg]	7.90[def]	29.56	4.0
USDA 110	Naringenin	T_0, T_2, T_6	11.02	49.91[efg]	7.30[def]	32.04	

[a] USDA 110 was inoculated at the rate of 10^6 cells per seed. Inhibitors were added at 10 μm concentrations and applied at planting (T_0) or 2 (T_2) or 6 (T_6) days after planting.

[b] Values are the means of 10 replicates. Others, Percentage of nodules which did not react with the two antibodies. Columns followed by the same letters (d through g) do not differ significantly ($P = 0.05$) for the indicated inoculum and inhibitor treatments. Nodule occupancy by USDA 110 and "others" was only affected by inoculation ($P = 0.05$).

[c] Mean of three application schedules.

variable if their stability is primarily determined by the rate of microbial degradation. Alternatively, the degree to which these compounds bind irreversibly to soil particles may vary with local differences in soil composition. Either mechanism could deplete the free concentration of these compounds in the soybean rhizosphere during the early stages of the infection process, when the degree of *nod* gene induction is most likely to affect competition between *B. japonicum* strains.

In *B. japonicum* USDA 110, USDA 138, USDA 123, USDA 127, USDA 129, and USDA 122, a broad spectrum of response to *nod* gene inhibitors was observed (Fig. 2). USDA 110 was the most resistant to *nod* gene inhibitors, while USDA 127 was the most sensitive. In *Rhizobium* species, patterns of *nodABC* response to a variety of plant flavonoids and exudates have been genetically linked to the *nodD* gene, which acts as a positive regulator of transcription of other *nod* genes (4, 6, 13, 15, 24, 28, 36, 37, 41). In *B. japonicum*, the extent to which variations in *nodD* genes between strains contributes to the response to plant flavonoids is unknown. USDA 123 contains two *nodD* genes, only one of which appears to be required for the induction of *nodC'-'lacZ* by daidzein, genistein, or soybean root extract (1). In the induction and inhibition experiments described here, all of the strains carried the USDA 123 *nodD* gene required for induction on pEA2-21, in addition to their own chromosomal *nodD* genes. Further studies are needed to elucidate the roles which these *nodD* genes play in the competitive and symbiotic properties of *B. japonicum* strains.

TABLE 3. Effect of inhibitor application on nodule occupancy in field-grown soybeans

Treatment[a]			Nodule occupancy (% of total)[b]					
Inoculum	Compound	Application	USDA 110	USDA 123	USDA 138	USDA 122	Others	Mixed
None	None	Liquid	3.6	50.3	8.3	0.6	30.2	7.0
None	Chrysin	Liquid	3.7	47.5	7.6	0.7	34.1	6.4
None	Naringenin	Liquid	1.8	46.0	11.1*	0.2	34.1	6.8
None	None	Peat	17.9	49.8	4.0	ND	22.6	5.7
None	Chrysin	Peat	17.9	50.0	5.6	ND	22.8	3.7
None	Naringenin	Peat	16.7	36.7	11.4	ND	31.4	3.8
USDA 110	None	Peat	33.5*	40.3	4.9	ND	17.0*	4.3
USDA 110	Chrysin	Peat	31.8*	42.3	1.6	ND	18.4*	5.9
USDA 110	Naringenin	Peat	27.4*	48.1	1.9	ND	18.8*	3.8
USDA 138	None	Peat	15.6	40.9	24.6*	ND	14.6*	4.3
USDA 138	Chrysin	Peat	10.2	45.3	24.4*	ND	16.1*	4.0
USDA 138	Naringenin	Peat	12.6	49.3	21.2*	ND	13.5*	3.4

[a] USDA 110 was inoculated at 4×10^7 cells per seed; USDA 138 was inoculated at 5×10^7 cells per seed. For liquid application, inhibitors (50 μM) were applied at a rate of 250 ml/ft at days 3, 4, and 5 after planting. For peat-based inoculum, inhibitors (100 μg per seed) and strains were mixed in at time of planting. Field site was located in Dane County, Wis.

[b] Treatments followed by an asterisk (*) were significantly different at ($P = 0.05$). Others, Percentage of nodules which did not react with the four antisera. ND, Not determined. Mixed, Percentage of nodules containing one or more of the four type strains.

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