Effects of *Rhizobium tropici*, *R. etli*, and *R. leguminosarum* bv. *phaseoli* on *nod* Gene-Inducing Flavonoids in Root Exudates of *Phaseolus vulgaris*

Maria Cristina Bolaños-Vásquez and Dietrich Werner

Fachbereich Biologie, Fg Angewandte Botanik und Zellbiologie, Philipps-Universität Marburg, Karl v. Frisch-Strasse, 35032 Marburg, Germany

Received 25 October 1996. Accepted 15 January 1997.

Flavonoids play an important role as signal molecules in the early stages of the legume-*Rhizobium* symbiosis. The flavonoid content in root exudates of individual seedlings of *Phaseolus vulgaris* cv. Rab39 was determined by a sensitive method that combines absorption onto cellulose acetate filter strips with separation, identification, and quantification of individual compounds by high-performance liquid chromatography/diode-array detector and gas chromatography-mass spectroscopy analyses. We identified six flavonoids, daidzein, coumestrol, naringenin, genistein, liquiritigenin, and isoliquiritigenin, the latter two of which had not previously been found in bean root exudate. Biological activities were demonstrated by induction of β -galactosidase activity in a *Rhizobium leguminosarum* bv. *phaseoli* strain containing a *nodC::lacZ* fusion controlled by the *nodD1* gene. Root exudate of common bean that had been inoculated with symbiotic *R. etli*, *R. leguminosarum* bv. *phaseoli*, or *R. tropici* contained more of the isoflavonoid daidzein, and the flavonoids naringenin, liquiritigenin, and isoliquiritigenin, than did exudate of plants that had not been inoculated. The relative concentrations of the signals depended on the nature of the bacterium. While daidzein, coumestrol, and naringenin were the dominant compounds in exudates of infected as well as uninfected seedlings, the daidzein and naringenin concentrations in particular were markedly enhanced after inoculation by all strains.

Phaseolus vulgaris L., a traditional crop in Latin America and one of the region's primary sources of dietary protein, forms N_2 -fixing root nodules in symbiotic association with *Rhizobium leguminosarum* bv. *phaseoli* (Jordan 1984), *R. etli* (Martinez-Romero et al. 1991), and *R. tropici* (Segovia et al. 1993). Establishment of the legume-*Rhizobium* symbiosis involves a complex developmental program. Formation of the new plant organ is based on several processes: rhizobia attach to the root hairs of their host and cause deformation and curling. Then the rhizobia invade the plant by newly formed tubular structures, the infection threads. Infections are initiated within the curl of the root hair (Lerouge et al. 1990; Truchet et

al. 1991). Concomitantly with the infection process, cells in the root cortex begin to divide and form the nodule primordium (Hirsch 1992). The coordination of these processes involves an intensive exchange of signal molecules between plant and microbe.

The initial signaling between the host plant and *Rhizobium* cells is mediated by flavonoids and isoflavonoids exuded from the plant root and present in the legume seed. They act as chemoattractants (Aguilar et al. 1988; Caetano-Anollés et al. 1992; Kape et al. 1991), and as inducers of rhizobial nodulation or *nod* genes. This process is mediated by the regulatory gene, *nodD*, and it leads to synthesis of nodulation factors by the bacterial symbiont. These return signals elicit root hair deformation, cortical cell division, and sometimes nodule formation by the legume (Banfalvi et al. 1988; Dénarié et al. 1992; Fisher and Long 1992; van Rhijn and Vanderleyden 1995).

Hungria et al. (1991) examined exudates from young roots of a black-seeded bean and identified three *nod* gene-inducing flavonoids: eriodictyol, the 7-*O*-glycoside of genistein, and naringenin. The concentrations of these compounds required to induce *nod* gene transcription in rhizobial strain 1283 were much higher than those needed in the alfalfa-*R. meliloti* system (Hartwig et al. 1990) but about equivalent to those needed in the soybean-*Bradyrhizobium japonicum* system (Kape 1992). Root exudates of 10-day-old seedlings from common bean inoculated with symbiotic *R. leguminosarum* bv. *phaseoli* bacteria contained more of the phytoalexin coumestrol and its isoflavonoid precursor daidzein than uninoculated controls did (Dakora et al. 1993). The phytoalexin coumestrol is the final product of the coumestan biosynthetic pathway, which synthesizes a number of compounds with functions in the bean-*R. leguminosarum* bv. *phaseoli* symbiosis. This pathway starts with simple, strongly chemotactic phenolics. Daidzein is a *nod* gene inducer and the isoflavonoid precursor of coumestrol.

Based on our knowledge of the intermediates of this pathway, we sought to determine if further compounds participate in the communication between rhizobia and bean. 2',4',4-Trihydroxychalcone (isoliquiritigenin) and 4',7-dihydroxyflavanone (liquiritigenin) are two of these intermediates. These two compounds have been found in a number of legumes, but have not yet been identified in bean root exudate (Wollenweber and Dietz 1981; Kape 1992). The objective of this study was to methodically characterize the symbiotic activities of root exu-

Corresponding author: D. Werner
E-mail: werner@mail.uni-marburg.de

dates from bean seedlings after inoculation with *R. etli*, *R. leguminosarum* bv. *phaseoli*, and *R. tropici*. We hypothesized that different *Rhizobium* spp. might influence the concentration of *nod* gene-inducing compounds in root exudates. Greater efficiency of the interaction between a particular strain and the root exudate compounds would allow that strain to out-compete others by initiating nodulation faster.

RESULTS

Detection of flavonoids in bean root exudate.

The system for cultivation of bean seedlings allows rapid preparation of the root exudate flavonoids from individual seedlings. High-performance liquid chromatography (HPLC) separation of the exudate with monitoring at 250 nm resolved a total of seven fractions (Fig. 1A). Absorbance measurements of peaks 1, 2, and 3 yielded no pronounced UV spectrum. Peak 4 produced a UV spectrum with $A_{\max}$ at 202, 249, and 301 nm, similar to that of daidzein (Mabry et al. 1970). The retention time for peak 4 on the standard HPLC gradient also was identical to that of authentic daidzein.

Absorbance measurements of peak 5 produced a UV spectrum with $A_{\max}$ at 213, 230, 275, and 313 nm, similar to that of authentic liquiritigenin: 212, 230, 276, and 312 nm. Absorbance measurements of asymmetrical peak 6 produced two UV spectra. The $A_{\max}$ of the first was 289 nm, with a shoulder at 327 nm. The second gave $A_{\max}$ at 240 and 345 nm. These closely matched spectra of authentic naringenin (289 and 326sh) and authentic coumestrol (240 and 345 nm). Absorbance measurements of peak 7 produced a UV spectrum with $A_{\max}$ at 248 and 365 nm, similar to that of authentic isoliquiritigenin (Kape et al. 1992). Retention times for all of the peaks on the standard HPLC gradient were identical to those of authentic standards.

All seven HPLC fractions were subjected to gas chromatography–mass spectroscopy (GC-MS) analyses as trimethylsilyl derivatives, and their GC and MS characteristics were compared with those of known reference standards (Table 1). The following fractions were recognized: fraction 4 as daidzein; fraction 5 as liquiritigenin; fraction 6A as naringenin; fraction 6B as coumestrol; and fraction 7 as isoliquiritigenin. Additionally, fraction 2, which was too small to yield a UV spectrum, was identified as genistein after treatment with a GC-MS concentration method. Co-chromatography with authentic standards in both analytical procedures confirmed the identification of

daidzein, liquiritigenin, coumestrol, naringenin, isoliquiritigenin, and genistein.

nod gene-inducing activities of compounds.

HPLC fractions 1 to 7 from individual seedlings induced *nod* gene transcription in *R. leguminosarum* bv. *phaseoli* and yielded 28, 400, 723, 650, 433, 410, and 592 Miller Units of β -galactosidase activity, respectively, for a representative uninoculated seedling and 118, 850, 809, 891, 636, 673, and 755 β -galactosidase units, respectively, for a seedling inoculated with *R. etli* Kim5s (Fig. 1B). Authentic standards at a concentration of 10 μ M induced the following β -galactosidase activities (mean of two measurements; β -galactosidase levels of the control bacteria that had not been exposed to flavonoids were subtracted from those of flavonoid-treated cultures): coumestrol (3,9 dihydroxycoumestran) 255 units; daidzein (4'-dihydroxyisoflavone) 484 units; 7'-hydroxyflavone 490 units; liquiritigenin (4'-dihydroxyflavanone) 566 units; 4'-dihydroxyflavone 625 units; isoliquiritigenin (2',4',4-trihydroxychalcone) 566 units; naringenin (4',5,7-trihydroxyflavanone) 727 units; eriodictiol (3',4',5,7-tetrahydroxyflavanone) 730 units, genistein (4',5,7-trihydroxyisoflavone) 1,332 units.

Quantitative tests with the six known flavonoids in root exudates were carried out, so that their relative importance in inducing *nod* genes of bean rhizobia could be assessed in replicated experiments. Although genistein had the highest $I_{\max}$ value and the lowest I_{50} value, daidzein, liquiritigenin, and isoliquiritigenin were also potent inducers (Fig. 2B). Naringenin was also active, but at somewhat higher concentrations, and coumestrol was essentially inactive (Fig. 2A). Increasing amounts of liquiritigenin or isoliquiritigenin were combined with daidzein to assess potential interaction between inducers. Figure 2C shows that the result was a synergistic interaction in both cases.

Rhizobium strains differentially modulate exudation of flavonoids by cv. Rab39.

We quantified *nod* gene-inducing flavonoids from root exudates of cv. Rab39 without rhizobia and after inoculation with eight different strains representative of the three *Rhizobium* spp. known to nodulate this legume. These data were analyzed statistically, and the results are summarized in Figure 3. Across all treatments, there was a high association ($r = 0.8$) between the exudation of liquiritigenin and isoliquiritigenin. Furthermore, the levels of daidzein and liquiritigenin ($r = 0.47$) and of daidzein and isoliquiritigenin ($r = 0.45$) in exudates were positively related.

Strain-specific effects on flavonoid inducers were clearly evident. *R. etli* TAL182, for example, was unable to elevate the level of any of the compounds, but both *R. etli* CIAT 2510 and *R. leguminosarum* bv. *phaseoli* CIAT 956 enhanced the concentrations of three of the five flavonoids. Conversely, levels of some of the compounds, especially liquiritigenin, were not influenced by rhizobia. At the other extreme, daidzein levels were significantly increased by five of the eight strains. It is noteworthy that none of the strains significantly reduced the concentration of any of the flavonoids (Fig. 3). Coumestrol concentrations were highly variable from seedling to seedling, and so no statistically significant differences could be detected by analysis of variance (ANOVA). There was merely a trend toward an accumulation after inoculation

Table 1. Identification of flavonoids^a

Authentic flavonoid	Fraction peak (no.) ^b	Base peak (m/z)	Rfit value compared with standard		
			Authentic flavonoid	Rab39 control	Rab39/Kim5s
Genistein	2	471	706	717	672
Daidzein	4	399	779	713	723
Liquiritigenin	5	400	716	755	759
Naringenin	6A	473	792	770	734
Coumestrol	6B	413	703	778	749
Isoliquiritigenin	7	457	773	661	668

^a Authentic flavonoids and peaks from exudates of Rab39 or Rab39 that had been inoculated with strain Kim5s were derivatized with trimethylsilyl and analyzed by gas chromatography–mass spectroscopy. Rfit values were calculated in comparison to a flavonoid databank. Rfit values greater than 600 imply identity with the known standard.

^b Identified by high-performance liquid chromatography.

with *R. etli* CIAT 2510, TAL 182, and *R. leguminosarum* bv. *phaseoli* CIAT 956 (Fig. 3).

After inoculation with *R. etli* strain Kim5s, CIAT 611, CIAT 2510, and *R. tropici* CIAT 899, a significant increase in daidzein content of the exudate of bean cv. Rab39 occurred; a significant increase in liquiritigenin content of exudate, however, was only evident after inoculation with *R. leguminosarum* bv. *phaseoli* strain CIAT 956 (Fig. 3). Naringenin content of root exudate was enhanced following inoculation with *R. etli* strains CIAT 609, CIAT 611, CIAT 2510, and H2C. The highest level of naringenin, achieved with *R. etli* strain CIAT 2510, was about three times that of the control (Fig. 3). Isoliquiritigenin concentration in the exudate showed a significant increase after inoculation with *R. etli* CIAT 2510 and *R. leguminosarum* bv. *phaseoli* CIAT 956. Independent assays revealed that inoculation with various *Rhizobium* strains consistently leads to an accumulation of daidzein, liquiritigenin, naringenin, and isoliquiritigenin, although to different extents. While daidzein, coumestrol, and naringenin are the major components in infected as well as uninfected seedling exudate, the levels of daidzein and naringenin gave more pronounced responses to inoculation.

DISCUSSION

The main focus of this study was to analyze root exudation of *nod* gene-inducing compounds in response to inoculation with a set of different *Rhizobium* spp., all of which nodulate a single legume. The interaction of bean with *R. etli*, *R. leguminosarum* bv. *phaseoli*, and *R. tropici* was ideal for this purpose. We selected a 22-h incubation time, because it is known that signal exchange between the symbiotic partners is intense during this period (Hirsch 1992). Bacteria were supplied at 10^7 per ml, a concentration approximating that in inocula (Vincent 1970).

We identified daidzein, liquiritigenin, coumestrol, naringenin, isoliquiritigenin, and genistein in root exudate from young seedlings of *Phaseolus vulgaris* L. 'Rab39'. All six signals were exuded in the absence of rhizobia and in the presence of three tested symbiotic species. All of these flavonoids are biosynthetically related (Fig. 4), and they were effective *nod* gene inducers, but to different extents. Coumestrol was a weak inducer, followed by daidzein, liquiritigenin, isoliquiritigenin, naringenin, and genistein in ascending order of inducer effectiveness. Two of these flavonoids, the chal-

Fig 1. High-performance liquid chromatography profile and *nod* gene-inducing activity of bean root exudate. **(A)** UV detection at 250 nm of exudate from a single bean seedling fractionated on a reverse-phase Hypersil column. Peaks identified: 2 as genistein, 4 as daidzein, 5 as liquiritigenin, 6 as naringenin and coumestrol, and 7 as isoliquiritigenin. **(B)** β -galactosidase activity induced by a *nodC::lacZ* gene fusion in *Rhizobium leguminosarum* bv. *phaseoli* strain 4292 (pIJ1737) (pIJ1730).

cone isoliquiritigenin and the corresponding flavanone liquiritigenin, have not been reported previously in root exudates from *P. vulgaris*. Both are precursors in the biosynthetic pathway of daidzein (Fig. 4), and they may perform special functions in the symbiosis. When increasing amounts of either of these flavonoids were added to a 3 μ M concentration of daidzein, *nod* gene expression increased significantly and synergistically. Similar effects on *nod* gene induction in *R. leguminosarum* bv. *phaseoli* have been described for eriodictyol and naringenin in combination with genistein (Hungria et al. 1992). Taken together, these findings suggest new interactions of isoflavonoids with flavanones, and of isoflavonoids with chalcone, perhaps at the same binding site.

Comparison of aglycones from bean root exudate with authentic standards that served as *nod* gene inducers shows that active compounds share free hydroxyl groups at the C4' and C7 positions. The C4 hydroxylation of chalcones is structurally analogous to the C7 hydroxylation of flavonoids (Fig. 4). Although a hydroxyl group in the C7 position could be a common binding site for stimulatory compounds in other rhizobial species (Györgypal et al. 1991; Kossak et al. 1987; Le Strange et al. 1990; Peters and Verma 1990; Zaat et al. 1989), hydroxyl groups at both C4' and C7 probably constitute common binding sites for those stimulatory rhizobia species that nodulate bean (*P. vulgaris*).

The *nod* gene-inducing activity of the flavonoids described here has already been described in other species (Maxwell et al. 1989; Recourt et al. 1991) and thus the flavonoid inducers of bean are not unique. Moreover, inoculation of *Vicia sativa* L. subsp. *nigra* roots with the compatible microsymbiont leads to an increased exudation of flavonoids into the rhizosphere (van Brussel et al. 1990; Spaik et al. 1991), and inoculation of soybean with the compatible microsymbiont or treatment with Nod factors leads to an accumulation of *nod* gene-inducing compounds (Schmidt 1994). Daidzein, coumestrol, genistein, and isoliquiritigenin are present in sterile soybean root exudate (d'Arcy-Lameta 1986; Kape et al. 1992; Schmidt 1994), and all four compounds induce *nod* genes in *B. japonicum* (Kossak et al. 1987; Kape et al. 1991, 1992).

The *nod* gene-inducing activity of isoliquiritigenin and liquiritigenin found in this study is consistent with previous reports showing such activity in soybean (Kape et al. 1992) and alfalfa (Maxwell et al. 1989; Hartwig et al. 1989). The synergistic effect produced by a chalcone and luteolin, a flavone, in *R. meliloti* (Hartwig et al. 1989) is comparable to that of isoliquiritigenin and the isoflavone daidzein in *R. leguminosarum* bv. *phaseoli*. Chalcones thus are synergistic both with flavones and isoflavones. An additive effect was also observed for daidzein and coumestrol (data not shown), which suggests that *R. leguminosarum* bv. *phaseoli* is not inhibited by this phytoalexin. A corresponding finding has previously been reported for *R. leguminosarum* bv. *viciae*, which was shown to grow in the presence of wyerone, a common phytoalexin in *V. faba* (Görge and Werner 1991).

The release of six *nod* gene-inducing flavonoids by bean roots suggests several possible ways that the plant may use to control the total *nod* gene-inducing activity in the rhizosphere. One mechanism could involve amplified signals, because bean is nodulated by three *Rhizobium* spp.. The plant may control *nod* gene-inducing signals by altering the levels of these compounds released from roots. Indeed, we found that more nodules were formed on seedlings of cv. Saxa when the rhizosphere was supplemented with isoliquiritigenin (data not shown).

Streit et al. (1992) tested the nodulation competitiveness of seven of the strains utilized in this work at pH 6.4 and 5.2. These strains were coinoculated with a Gus⁺ mutant of *R. etli* strain Kim5s, and a range of nodulation competitiveness was observed. Our results indicate that the observed flavonoid accumulation is not primarily related to competitiveness. For example, *R. etli* CIAT 2510 is a poor competitor (Streit et al. 1992), yet it has strong positive effects on flavonoid accumulation. Inoculating bean roots with an infective strain of *R. etli*, *R. leguminosarum* bv. *phaseoli*, or *R. tropici* increased the total *nod* gene-inducing activity of root exudate, and this effect was primarily due to increases in four flavonoids: daidzein, liquiritigenin, naringenin, and isoliquiritigenin, although to different extents. After 22 h of incubation with these

Fig 2. (A,B) Induction of *nodC* in *Rhizobium leguminosarum* bv. *phaseoli* strain 4292(pIJ1737)(pIJ1730) by flavonoids known to be exuded by bean roots. **(C)** Interaction between daidzein and liquiritigenin or isoliquiritigenin as inducer. Daidzein was added at a concentration of 3 μ M; the resulting basal level of induction is given as a shaded horizontal line. Mean values from three trials are shown.

strains, markedly enhanced levels of daidzein and isoliquiritigenin could already be seen with some strains. If one considers the synergistic effects between these two substances as well as the fact that the flavonoid that exhibits the most potent *nod* gene activating effects when considered alone, namely genistein, was only present in very small quantities at this stage of incubation, it becomes clear that both the interactions between various flavonoids and the time course of exudation

in connection with nodulation need to be studied much more closely in the future.

MATERIALS AND METHODS

Growth of seedlings and preparation of root exudate.

Bean seeds (*Phaseolus vulgaris* L. 'Rab 39', CIAT International Agriculture Center, Colombia) were surface sterilized

Fig 3. Amounts of five flavonoids exuded by single bean roots (*Phaseolus vulgaris* L. 'Rab39') 22 h after incubation with 5 mM morpholinoethane sulfonic acid buffer, pH 6.8 plus 1 mM CaCl_2 (MES- CaCl_2 ; control), or MES- CaCl_2 containing rhizobia (10^7 CFU per ml per plant). All strains except TAL 182 are from the CIAT collection. Each value represents mean of five seedlings. Columns sharing same letter are not significantly different ($P = 0.05$).

for 3 min in 95% ethanol, followed by 3 min in 3% commercial bleach, and then washed five times with sterile water. Thereafter, they were soaked in sterile water for 30 min and then washed again with sterile water five times. Seedlings were germinated on sterile paper for 40 h at 25°C in the dark, and

then individually transferred into 2.3-ml test tubes. The tubes contained cellulose acetate filter strips (1.0 × 6.5 cm, Type OE66, Schleicher and Schüll, Göttingen, Germany) and either 5 mM MES (morpholinoethane sulfonic acid, Serva, Heidelberg, Germany) buffer, pH 6.8 plus 1 mM CaCl₂ (MES-CaCl₂), or

Fig 4. Structures of daidzein, liquiritigenin, naringenin, coumestrol, genistein, and isoliquiritigenin, the substances identified by high-performance liquid chromatography as peaks 2, 4, 5, 6, and 7, respectively. Biosynthetic relationships of compounds are indicated.

suspensions of the bacteria. Seedlings were incubated under the following conditions in a growth cabinet: 25°C, relative humidity 75%, 14h/10h light/dark period, irradiance 13W m⁻². After an incubation period of 22 h, the filter strips were rinsed four times with distilled water to remove all highly water-soluble compounds and cell debris. Flavonoids were extracted with two sequential rinses with 1 ml of methanol and one with 90% methanol. Extracts were gradually evaporated under vacuum to dryness and stored at -20°C prior to use.

Bacteria and culture conditions.

R. etli strains Kim5s, CIAT 609, CIAT 611, CIAT 2510, and H2C, *R. leguminosarum* bv. *phaseoli* CIAT 956, and *R. tropici* CIAT 899 were provided by the International Center of Tropical Agriculture-CIAT, Cali, Colombia. *R. etli* TAL182 was from Canada. Bacteria were grown at 28°C on a rotary shaker in YM medium (Vincent 1970). Cells were centrifuged for 5 min at *g* and resuspended in sterile MES-CaCl₂ prior to inoculation of plants.

HPLC.

Extracts of dried material were redissolved in 25 µl of methanol/dimethylformamide (19:1, vol/vol). A 20-µl portion of the solution was injected into an HPLC system equipped with an ODS-Hypersil column (5 µm, 250 × 4 mm, Hewlett Packard, Böblingen, Germany) and separated at a flow rate of 1 ml per min with the following gradient: 10 to 55% solvent B (acetonitrile) within 25 min, solvent A (water acidified to pH 3.3 with acetic acid), and 5 min of isocratic chromatography with 55% acetonitrile. Eluted compounds were monitored and analyzed with a diode-array-detector (195 to 365 nm, Spectra Focus, Spectra-Physics, San Jose, CA). Fractions of HPLC runs were recollected and dried under vacuum for analytical (GC-MS) and biological studies. After tentative identification, comparisons with commercial standards were carried out employing all above analyses as well as co-chromatography. Flavonoids present in root exudates were quantified by integrating HPLC peaks, using the following wavelengths (nm): 250 nm for daidzein, 275 nm for liquiritigenin, 290 nm for naringenin, 345 nm for coumestrol, and 365 nm for isoliquiritigenin. The concentrations of the flavonoids were determined with known quantities of authentic standards, by applying the integrator function of the Spectra Physics software package.

GC-MS.

Dried fractions or purified compounds were derivatized by incubation for 15 h at 60°C with 99 µl of N₂O-bis-(trimethylsilyl)trifluoroacetamide plus 1 µl of trimethylchlorosilane (TMCS, Sigma, Munich, Germany). The separation was accomplished on a DB-5 column (30 m × 0.25 mm ID) 5% phenylpolysiloxane and 95% methylpolysiloxane (J&W Scientific, Folsom, CA) under the following conditions: helium pressure 15 lb/in², injector temperature 280°C, GC-temperature program 70 to 310°C at 5°C min⁻¹. The mass was determined at a scan 200 to 650 a.m.u. per nominal second. When the GC-MS analysis of the fraction did not show any peaks, fractions were concentrated under nitrogen, resuspended in a volume of 10 µl of chloroform, and again injected into GC-MS with the same temperature program. Peaks were identified per computer search of user-generated reference libraries and by comparison with commercial standards.

Biological activities.

The *nod* gene-inducing capacity of commercial standards or fractions was assayed as β-galactosidase activity transcribed from a *nodC::lacZ* fusion under the control of *nodD1* gene in *R. leguminosarum* bv. *phaseoli* 4292 (pIJ1737)(pIJ1730) (Davis and Johnston 1990). Fractions of two HPLC runs were pooled, dried under vacuum, and assayed for their *nod* gene-inducing capacity as described (Kape et al. 1991), except that the bacteria were grown on YM medium (Vincent 1970) and *o*-nitrophenyl-β1,4-galactoside (Sigma, Munich, Germany) was used as a substrate (Miller 1972).

Statistics.

Flavonoids were analyzed statistically by ANOVA (Norusis 1992; Bortz 1989). When the ANOVA showed significant treatment effects, the least significant difference (LSD) test, or Bonferroni procedure, was applied to make comparisons among means at the α = 0.05 level of significance (Norusis 1992; Winer et al. 1991).

ACKNOWLEDGMENTS

We thank S. Pueppke and W. Streit for constructive discussions. This work was supported by a grant from Deutscher Akademischer Austauschdienst (DAAD) and by the Sonderforschungsbereich 395 Interaktion, Anpassung und katalytische Fähigkeiten von Bodenmikroorganismen (Project A4).

LITERATURE CITED

- Aguilar, J. M. M., Ashby, A. M., Richards, A. J. M., Loake, G. J., Watson, M. D., and Shaw, C. H. 1988. Chemotaxis of *Rhizobium leguminosarum* bv. *phaseoli* towards flavonoid inducers of the symbiotic nodulation genes. J. Gen. Microbiol. 134:2741-2746.
- Banfalvi, Z., Nieuwkoop, A., Schell, M., Besl, L., and Stacey, G. 1988. Regulation of *nod* gene expression in *Bradyrhizobium japonicum*. Mol. Gen. Genet. 214:420-424.
- Bortz, J. 1989. Statistik für Sozialwissenschaftler. 3rd ed. Springer Verlag, Berlin.
- Caetano-Anollés, G., Wrobel-Boerner, E., and Bauer, W. D. 1992. Growth and movement of spot inoculated *Rhizobium meliloti* on the root surface of alfalfa. Plant Physiol. 98:1181-1189.
- Dakora, F. D., Joseph, C. M., and Phillips, D. A. 1993. Common bean root exudates contain elevated levels of daidzein and coumestrol in response to *Rhizobium* inoculation. Mol. Plant-Microbe Interact. 6: 665-668.
- d'Arcy-Lameta, A. 1986. Study of soybean and lentil root exudates. II: Identification of some polyphenolic compounds, relation with plantlet physiology. Plant Soil 92:113-123.
- Davis, E. O., and Johnston, A. W. B. 1990. Regulatory function of the three *nodD* genes of *Rhizobium leguminosarum* bv. *phaseoli*. Mol. Microbiol. 4:933-941.
- Dénarié, J., Debellé, F., and Rosenberg, C. 1992. Signaling and host range variation in nodulation. Annu. Rev. Microbiol. 46:497-531.
- Fisher, R. F., and Long, S. R. 1992. *Rhizobium*-plant signal exchange. Nature 357:655-660.
- Görge, E., and Werner D. 1991. Degradation of wyerone, the phytoalexin of faba beans by *Rhizobium leguminosarum*. Curr. Microbiol. 23:153-157.
- Györgypal, Z., Kondorosi, E., and Kondorosi, A. 1991. Diverse signal sensitivity of NodD protein homologs from narrow and broad host range rhizobia. Mol. Plant-Microbe Interact. 4:356-364.
- Hartwig, U. A., Maxwell, C. A., Joseph, C. M., and Phillips, D. A. 1989. Interactions among flavonoid *nod* gene inducers released from alfalfa seeds and roots. Plant Physiol. 91:1138-1142.
- Hartwig, U. A., Maxwell, C. A., Joseph, C. M., and Phillips, D. A. 1990. Effects of alfalfa *nod* gene inducing flavonoids on *nodABC* transcription in *Rhizobium meliloti* strains containing different *nodD* genes. J.

- Bacteriol. 172:2769-2773.
- Hirsch, A. M. 1992. Developmental biology of legume nodulation. New Phytol. 122:211-237.
- Hungria, M., Johnston, A. W. B., and Phillips, D. A. 1992. Effects of flavonoids released naturally from bean (*Phaseolus vulgaris*) on *nodD*-regulated gene transcription in *Rhizobium leguminosarum* bv. *phaseoli*. Mol. Plant-Microbe Interact. 5:199-203.
- Hungria, M., Joseph, C. M., and Phillips, D. A. 1991. *Rhizobium nod* gene inducers exuded naturally from roots of common bean (*Phaseolus vulgaris* L.). Plant Physiol. 97:759-764.
- Jordan, D. C. 1984. Family III, Rhizobiaceae. Pages 234-242 in: Bergey's Manual of Systematic Bacteriology. Vol. 1. N. R. Krieg and J. G. Hoh, eds. Williams & Wilkins, Baltimore.
- Kape, R. 1992. Phenolic Signalsubstanzen in der Symbiose von *Bradyrhizobium japonicum*. Ph.D. diss. Fachbereich Biologie der Universität Marburg, Marburg, Germany.
- Kape, R., Parniske, M., Brand, S., and Werner, D. 1992. Isoliquiritigenin a strong *nod* gene- and glyceollin resistance inducing flavonoid from soybean root exudate. Appl. Environ. Microbiol. 58:1705-1710.
- Kape, R., Parniske, M., and Werner, D. 1991. Chemotaxis and *nod* gene activity of *Bradyrhizobium japonicum* in response to hydroxycinnamic acids and isoflavonoids. Appl. Environ. Microbiol. 57:316-319.
- Kosslak, R. M., Bookland, R., Barkei, J., Paaren, H. E., and Appelbaum, E. R. 1987. Induction of *Bradyrhizobium japonicum* common *nod*-genes by isoflavonoids isolated from *Glycine max*. Proc. Natl. Acad. Sci. USA 84:7428-7432.
- Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C., and Dénarié, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 344:781-784.
- Le Strange, K. K., Bender, G. L., Djordjevic, M. A., Rolfe, B. G., and Redmond, J. W. 1990. The *Rhizobium* strain NGR234 *nodD1* gene product responds to activation by simple phenolic compounds vanillin and isovanillin present in wheat seedling extracts. Mol. Plant-Microbe Interact. 3:214-220.
- Mabry, T. J., Markham, K. R., and Thomas, M. B. 1970. Systematic Identification of Flavonoids. Springer Verlag, New York.
- Martinez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P., and Pardo, M. A. 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. 41:417-426.
- Maxwell, C. A., Hartwig, U. A., Joseph, C. M., and Phillips, D. A. 1989. A chalcone and two related flavonoids released from alfalfa roots induce *nod* genes of *Rhizobium meliloti*. Plant Physiol. 91:842-847.
- Miller, J. H. 1972. Pages 352-355 in: Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Norusis, M. J. 1992. SPSS for Windows. SPSS, Chicago.
- Peters, N. K., and Verma, D. P. S. 1990. Phenolic compounds as regulators of gene expression in plant-microbe interactions. Mol. Plant-Microbe Interact. 3:4-8.
- Recourt, K., Schripsema, J., Kijne, J. W., van Brussel, A. A. N., and Lugtenberg, B. J. J. 1991. Inoculation of *Vicia sativa* subsp. *nigra* roots with *Rhizobium leguminosarum* bv. *viciae* results in release of *nod* gene activating flavanones and chalcones. Plant Mol. Biol. 16: 841-852.
- Scheidemann, P. 1994. Identifizierung und Charakterisierung von Flavonoiden im Wurzelexsudat von *Robinia pseudoacacia*. M.Sc. diss. Fachbereich Biologie der Universität Marburg, Marburg, Germany.
- Schmidt, P. E. 1994. Akkumulation von Flavonoiden als frühes symbiotisches Symptom in der *Glycine max/Rhizobium* Symbiose. Ph.D. diss. Fachbereich Biologie der Philipps-Universität Marburg, Marburg, Germany.
- Segovia, L., Young, J. P. W., and Martinez-Romero, E. 1993. Reclassification of American *Rhizobium leguminosarum* bv. *phaseoli* type I strains as *Rhizobium etli* sp. nov. Int. J. Syst. Bacteriol. 43:374-377.
- Spaink, H. P., Sheeley, D. M., van Brussel, A. A. N., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and Lugtenberg, B. J. J. 1991. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 354:125-130.
- Streit, W., Kosch, K., and Werner, D. 1992. Nodulation competitiveness of *Rhizobium leguminosarum* bv. *phaseoli* and *Rhizobium tropici* strains measured by glucuronidase (*gus*) gene fusion. Biol. Fertil. Soils 14:140-144.
- Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Promé, J. C., and Dénarié, J. 1991. Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature 351:670-673.
- van Brussel, A. A. N., Recourt, K., Pees, E., Spaink, H., Tak, T., Wijffelman, C. A. T., Kijne, J., and Lugtenberg, B. J. J. 1990. A biovar-specific signal of *Rhizobium leguminosarum* bv. *viciae* induces increased nodulation gene-inducing activity in root exudate of *Vicia sativa* subsp. *nigra*. J. Bacteriol. 172:5394-5401.
- van Rhijn, P., and Vanderleyden J. 1995. The *Rhizobium*-plant symbiosis. Microbiol. Rev. 59:124-142.
- Vincent, J. M. 1970. Nitrogen Fixation in Legumes. Academic, New York.
- Winer, B. J., Brown, D. R., and Michaels, K. M. 1991. Statistical Principles in Experimental Design. 3rd ed. McGraw-Hill, New York.
- Wollenweber, E., and Dietz V. H. 1981. Occurrence and distribution of free flavonoid aglycones in plants. Phytochemistry 20:869-932.
- Zaat, S. A. J., Schripsema, J., Wijffelman, C. A., van Brussel, A. A. N., and Lugtenberg, B. J. J. 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.