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Isoliquiritigenin, a Strong *nod* Gene- and Glyceollin Resistance-Inducing Flavonoid from Soybean Root Exudate

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Isoflavonoid signal molecules from soybean (*Glycine max* (L.) Merr.) seed and root exudate induce the transcription of nodulation (*nod*) genes in *Bradyrhizobium japonicum*. In this study, a new compound with symbiotic activity was isolated from soybean root exudate. The isolated 2',4',4-trihydroxychalcone (isoliquiritigenin) is characterized by its strong inducing activity for the *nod* genes of *B. japonicum*. These genes are already induced at concentrations 1 order of magnitude below those required of the previously described isoflavonoid inducers genistein and daidzein. Isoliquiritigenin is also a potent inducer of glyceollin resistance in *B. japonicum*, which renders this bacterium insensitive to potentially bactericidal concentrations of glyceollin, the phytoalexin of *G. max*. No chemotactic effect of isoliquiritigenin was observed. The highly efficient induction of *nod* genes and glyceollin resistance by isoliquiritigenin suggests the ecological significance of this compound, although it is not a major flavonoid constituent of the soybean root exudate in quantitative terms.

The interaction between legumes and bacteria of the genera *Rhizobium* and *Bradyrhizobium* results in the formation of nitrogen-fixing root nodules. One early event in this interaction is the induction of rhizobial nodulation genes (*nod* genes), which is mediated via the *nodD* gene product (for a review, see references 14 and 17). As a result of the *nod* gene induction, a nodulation factor (Nod factor) is produced by the bacterial symbiont, which in turn elicits root hair deformation, cortical cell devision, and nodule formation by the legume (32). Besides the well-characterized flavonoid inducers in all rhizobial systems (for a review, see references 17 and 29), further inducing compounds not belonging to this class of phenolic compounds have been identified by recent work; e.g., for *Bradyrhizobium japonicum*, a broad spectrum of diverse inducers was characterized (4, 13).

Analysis of soybean root exudate resulted in the identification of coumestrol and daidzein (5). In root extracts, genistein was found in addition to daidzein and coumestrol (25). All three compounds induce the *nod* genes of *B. japonicum* (15).

Besides the well-established *nod* gene-inducing activity of the flavonoids, further effects of these compounds on the microsymbiont are of symbiotic relevance (24). For fast-growing rhizobia, e.g., *Rhizobium meliloti* (3), a chemotactic effect of flavonoids was found, while *B. japonicum* did not respond (13) or responded only very weakly (1) to isoflavonoids. The existence of a correlation between the *nod* gene-inducing and the chemotaxis-eliciting activities of the flavonoids and isoflavonoids was judged by these authors as being different for the different systems.

The list of the physiological effects of flavonoids on rhizobia is still growing, as exemplified by the discovery that the incubation of soybean-nodulating rhizobia (*B. japonicum* and *Sinorhizobium fredii*) in the presence of the isoflavone genistein or daidzein induced resistance to potentially bactericidal concentrations of glyceollin, the phytoalexin of soybean (22). This effect of isoflavonoids was found to be independent of *nodD*. Experiments dealing with phytoalexin-containing root exudate suggest an important role of isoflavonoid-inducible glyceollin resistance for the survival of rhizobia in the rhizosphere of the soybean roots (22).

The phytoalexin glyceollin is the final product of the pterocarpan biosynthetic pathway (9), which includes a number of compounds with functions in *B. japonicum*-*Glycine max* symbiosis. This pathway starts with simple phenolic compounds which exhibit a strong chemotactic effect and a weak *nod* gene-inducing effect. Daidzein, a precursor of glyceollin (9), is a strong *nod* gene inducer but lacks any chemotactic activity (13). On the basis of knowledge of intermediates in this pathway, we aimed to find out whether other compounds participate in the communication between *B. japonicum* and *G. max*. 2',4',4-Trihydroxychalcone (isoliquiritigenin) is one of these intermediates. The objective of this study was to characterize the symbiotic activities and the ecological relevance of isoliquiritigenin. This chalcone was found in a number of legumes (for a review, see reference 34). Recently, Recourt et al. (26) identified it among other flavanones and chalcones in the root exudate of *Vicia sativa* subsp. *nigra* after inoculation with *Rhizobium leguminosarum* bv. *viciae*, but isoliquiritigenin has not yet been identified in soybean root exudate.

MATERIALS AND METHODS

Synthesis of isoliquiritigenin. Preliminary experiments were conducted with a sample of isoliquiritigenin which was kindly provided by E. Wollenweber (Darmstadt, Germany). Further experiments were performed with material synthesized in our laboratory.

Isoliquiritigenin was synthesized by aldol condensation of resacetophenone (2',4'-dihydroxyacetophenone; Sigma, Munich, Germany) and 4-hydroxybenzaldehyde (Sigma) under nitrogen (6, 20, 35). Both the ketone (resacetophenone) and the aldehyde (4-hydroxybenzaldehyde) were dissolved together in 15 ml of ethanol at a concentration of 25 mM. Potassium hydroxide (50 ml of a 60% [wt/vol] solution) was added dropwise. The mixture was chilled in an ice bath to maintain the temperature below 4°C. Afterwards, the reaction mixture was stirred for 2 more days under the exclusion of oxygen. Purification of isoliquiritigenin was accomplished

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by column chromatography on silica gel (Macherey-Nagel, Düren, Germany) (chloroform-methanol [94:6, vol/vol]) and polyamide (Roth, Karlsruhe, Germany) (methanol). The yield of crude product was 31.2% of the primary reactants. Recrystallization from methanol-water resulted in isoliquiritigenin with a melting point of 202°C (202 to 204°C [6]). A molecular mass of 256.073 for isoliquiritigenin was calculated. In mass spectroscopy, *m/e* 256 was found. The identity of the synthesized isoliquiritigenin was confirmed by ^{1}H NMR: CD_3OD δ 7.79 (1H, d, $J_{\alpha,\beta}$ = 16 Hz, H_α), 7.61 (1H, d, $J_{\beta,\alpha}$ = 16 Hz, H_β), 7.62 (2H, dt, J = 9 Hz, 2 and 6 H), 6.88 (2H, dt, J = 9 Hz, 3 and 5 H), 7.98 (1H, d, $J_{6',5'}$ = 9 Hz, 6'H), 6.42 (1H, dd, $J_{5',6'}$ = 9 Hz, $J_{5',3'}$ = 3 Hz, 5'H), 6.29 (1H, d, $J_{3',5'}$ = 3 Hz, 3'H), $(CD_3)_2$ SO δ 3.39 (1H, s, 4OH), 10.41 (1H, s, 4'OH), 13.63 (1H, s, 2'OH).

Preparation of soybean root exudate. Soybean seeds (*G. max* (L.) Merr. cv. Maple Arrow) were washed with water and afterwards surface sterilized for 10 min in 30% hydrogen peroxide plus some drops of Tween 20 (polyoxyethene sorbitan monolaurate; Serva, Heidelberg, Germany). After sterilization, seeds were washed 10 times, and afterwards they imbibed sterile tap water for 7 h on a shaker (100 rpm) at 28°C. The water was changed twice during this procedure to remove seed-derived compounds. After soaking, seeds were again rinsed thoroughly with sterile water and grown in sterile Perlite-Vermiculite (1:2) for 65 h in a growth cabinet (25°C; relative humidity, 75%; 16-8 h light-dark period, irradiance, 13 W m^{-2}). Then the seedlings were individually transferred onto a stainless-steel lattice which was placed in a glass petri dish (diameter, 22 cm; height, 7 cm). The roots of the seedlings grew through the holes of the lattice and along the surface of a cellulose acetate filter (diameter, 203 mm, type OE66; Schleicher and Schuell GmbH, Dassel, Germany) at the bottom of the petri dish 1 cm below the lattice. The filter was moistened with MES [2-(*N*-morpholino)ethanesulfonic acid] buffer (5 mM, pH 6.2) (Serva) plus $CaCl_2$ (1 mM) (MES-$CaCl_2$). The cellulose acetate filter absorbs the flavonoids exuded by the root (27). After 4 days, the filter was rinsed three times with distilled water to remove all easily water-soluble compounds and cell debris. Flavonoids were extracted with three washes of methanol. After filtration (glass fiber filter, Whatman GF/C), the crude extract was stored at −20°C in the dark. It was used for analytical studies and the preparation of isoliquiritigenin.

Preparation of soybean seed exudate. Soybean seed exudate was produced as follows. Seeds were washed and sterilized as described above for the preparation of root exudate. Seeds were then placed on sterile cellulose acetate filters moistened with MES-$CaCl_2$ for 24 h. Flavonoid preparation was performed as described above for the root exudate.

Analytical methods. (i) HPLC. High-pressure liquid chromatography (HPLC) was performed on an RP 18 (Octadecyl) 10-μm column (250 by 4.6 mm) (Serva). The following gradient was used for the separation of isoliquiritigenin from compounds with similar retention behavior at a flow rate of 1 ml/min: 27 to 32% solvent B (acetonitrile) within 50 min; solvent A, water acidified to pH 3 with acetic acid. Eluting compounds were detected with a variable-wavelength detector at 250 or 368 nm (absorption maximum of isoliquiritigenin). For further analytical and biological studies, fractions of several HPLC runs containing the putative isoliquiritigenin were pooled and dried under vacuum. These pooled fractions will be termed the isoliquiritigenin fraction. Flavonoid compounds of the soybean root exudate which have already been described in the literature (daidzein, genistein, and coumestrol) were identified by cochromatography with authentic standards.

(ii) TLC. Thin layer chromatography (TLC) was executed on nano plates (Sil-20 UV254) (10 by 10 cm) (Macherey-Nagel). The best separation of the isoflavonoids was obtained with toluene-ethylacetate-methanol (70:25:5, vol/vol/vol). TLC plates were evaluated with a Desaga CD60 densitometer (Desaga, Heidelberg, Germany), which allows UV-visible absorption spectra of single spots on TLC plates.

(iii) GC-MS. For gas chromatography-mass spectroscopy (GC-MS) analysis, a Varian 3400 in combination with an Ion Trap Detector ITD 800 (Finnigan MAT, Bremen, Germany) was used. The separation was accomplished on a DB-1 column (30 m) (J & W Scientific) with the following temperature program: 220 to 280°C at 2°C/min, after an initial delay of 1 min. The final column temperature was held for 15 min. Samples were purified by HPLC before they were analyzed by GC-MS. These purified samples were dried under vacuum and stored until derivatization over silica gel. The dry samples were taken up in 99% bis(trimethylsilyl)trifluoroacetamide–1% trimethylchlorosilane (Sigma) and incubated at 60°C for 15 h to obtain the trimethylsilyl derivatives of the flavonoids.

Biological activities of isoliquiritigenin. (i) Chemotaxis. The chemotaxis assay was performed essentially as described previously (13). The following modifications were introduced. *B. japonicum* 110*spc*4 (28) was cultured in the complex medium 20E (33) supplemented with MES (20 mM, pH 6.5) (20E-MES). Cultures were incubated at 28°C on a rotary shaker (100 rpm). Isoliquiritigenin was tested at concentrations from 10^{-4} to 10^{-10} M. Caffeic acid, a strong chemoattractant (13) of *B. japonicum*, was used as a positive control. Chemotactic activity was expressed as the chemotactic ratio (the number of bacteria attracted by the test substance divided by the number of bacteria in the buffer control).

(ii) Induction of *nod* genes. The assay of *nod* gene induction was performed according to Kape et al. (13). The following changes were introduced. *B. japonicum* 110*spc*4 harboring plasmid pRJ458 (7) was grown in 20E-MES. Cultures were incubated at 28°C on a rotary shaker (100 rpm) for 15 h in the presence of varying concentrations of the test compounds (10^{-4} to 10^{-10} M). For *nod* gene induction tests with isoliquiritigenin isolated from root exudate, the culture volume was reduced to 0.5 ml. Cells were grown in 2-ml glass vials with Teflon-lined screw caps (Renner GmbH, Dannstadt, Germany).

(iii) Induction of glyceollin resistance. The glyceollin resistance induction assay was conducted as described previously (22). In short, the rhizobial strain (here, *B. japonicum* 110*spc*4 harboring plasmid pRJ458) was cultured in a complex medium in the presence of the test compound (here, 10^{-5} M isoliquiritigenin). After 15 h, an aliquot of this culture was transferred to MES buffer (5 mM, pH 6.5) containing glyceollin (3×10^{-4} M). After 3 h of incubation in this phytoalexin solution, the appropriate dilutions were plated on 20E agar to estimate the number of surviving CFU. Glyceollin resistance is expressed as the percentage of cells that survived the incubation in glyceollin.

Comparison of the symbiotic effects of the intermediates of the pterocarpan biosynthetic pathway. *nod* gene induction and induction of glyceollin resistance by intermediates of the pterocarpan biosynthetic pathway and side branches of this pathway were tested together as follows. *B. japonicum* 110*spc*4 harboring pRJ458 was cultured in 20E-MES as described for the *nod* gene induction assay in the presence of

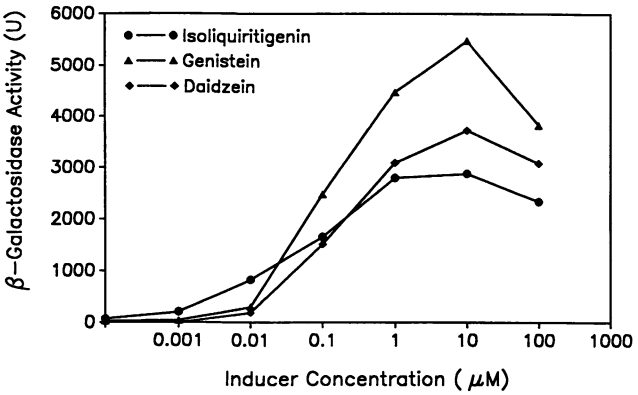

FIG. 1. Induction of *B. japonicum nod* genes by isoliquiritigenin, genistein, and daidzein. Compounds were tested for their abilities to induce transcription of β-galactosidase from *B. japonicum* 110*spc*4 harboring plasmid pRJ458 (*nodC-lacZ*). The background level of β-galactosidase activity was subtracted.

the test compounds (10^{-5} M). After 15 h, samples of these cultures were tested for *nod* gene induction and induction of glyceollin resistance. Chemotaxis data presented in this study, except those for isoliquiritigenin, were taken from Kape et al. (13).

RESULTS

Biological activity of isoliquiritigenin. (i) Induction of *nod* genes. Isoliquiritigenin was identified as a strong *nod* gene inducer. This induction was compared to that of other *nod* gene inducers (Fig. 1). Isoliquiritigenin exhibited an inducing activity already at 1 nM. This concentration is about 1 order of magnitude below those required of the previously described strong *nod* gene inducers daidzein and genistein. The stronger *nod* gene-inducing activity of isoliquiritigenin at extremely low concentrations is in contrast to its reduced inducing activity at higher concentrations; the opposite is true for daidzein or genistein. The maximal *nod* gene induction of all three compounds was achieved at a concentration of 10 μM. The concentrations required for half-maximum activities of *nod* gene induction were 52, 144, and 159 μM for isoliquiritigenin, daidzein, and genistein, respectively (mean values of two experiments).

(ii) Chemotactic effect of isoliquiritigenin. No chemotactic effect of isoliquiritigenin could be detected, while the positive control, caffeic acid, caused a strong chemotactic response. A chemotactic ratio of 31 was obtained for caffeic acid at a concentration of 10 mM (mean value of three independent experiments).

(iii) Induction of glyceollin resistance. Isoliquiritigenin proved to be a strong inducer of glyceollin resistance in *B. japonicum* 110*spc*4 harboring pRJ458. As depicted in Fig. 3, the applied concentration of 10 μM resulted in a resistance of 90% of all cells in the test culture against a glyceollin concentration of 300 μM. The degree of resistance is as high as that obtained by genistein, the best inducer of glyceollin resistance described up to now (22).

Detection of isoliquiritigenin in soybean root exudate. The applied system for cultivation of soybean seedlings allows fast preparation of the root exudate flavonoid fraction without any contaminations from the seed exudate flavonoid fraction. The following experimental evidence proves the presence of isoliquiritigenin in this root exudate flavonoid fraction.

HPLC analysis with peak detection at 250 nm allowed the identification of the major flavonoid compounds in the root exudate, namely, daidzein (retention time, 11.3 min), genistein (retention time, 21.8 min), and coumestrol (retention time, 23.2 min). Peak detection at 368 nm, the maximum absorption of isoliquiritigenin, and cochromatography with the authentic standard (retention time, 34.4 min) provided strong evidence for the presence of this chalcone in the root exudate. Figure 2 illustrates the HPLC separation with detection at both wavelengths. The HPLC fraction containing the putative isoliquiritigenin was submitted to further testing by using analytical methods.

GC-MS analysis exhibited identical retention times for the trimethylsilyl derivatives of the main constituent in the isoliquiritigenin fraction and the authentic standard (retention time, 22 min). The calculated molecular mass of the trimethylsilyl derivative of isoliquiritigenin is 472.19. The *m/e* 472 was found for the authentic standard as well as for the main component of the isoliquiritigenin fraction. In both spectra, the base peak was *m/e* 458. These consistent data for the authentic standard and the isolated sample are strong arguments for their identity.

A separation of the isoliquiritigenin fraction on thin-layer plates resulted in a main spot with an R_f value of 0.48, identical to that for the authentic standard. The UV-visible spectra (200 to 600 nm) of these spots were almost identical, with absorption maxima at 378 nm. Minor differences occurred in the short wavelength area which were probably due to overlying contaminating substances from the root exudate.

Further important confirming evidence that the main component of the isoliquiritigenin fraction is in fact isoliquiritigenin came from the *nod* gene induction assay. Both the authentic standard and the isoliquiritigenin fraction, adjusted to the same A_{368}, exhibited almost identical concentration dependence curves of *nod* gene induction, with the typical onset of *nod* gene induction at concentrations below 10 nM.

A quantitative analysis of the identified *nod* gene inducers in soybean root exudate by HPLC resulted in concentration ratios for daidzein-genistein-coumestrol-isoliquiritigenin of 28.6:2.3:3.3:1. There was hardly any variation in this ratio for three separate seedling cultivations. Hence, daidzein is the quantitatively dominant *nod* gene inducer in the exudate.

Composition of soybean seed exudate. HPLC analysis of soybean seed exudate resulted in the detection of high quantities of daidzein and genistein, while isoliquiritigenin and coumestrol were missing in the seed exudate.

Comparison of the symbiotic activities of intermediates of the pterocarpan biosynthetic pathway. Figure 3 summarizes the effects of the intermediates of the pterocarpan pathway and its side branches on *B. japonicum*. Striking differences between the individual intermediates in relation to their symbiotic effects are apparent. While only simple phenolic acids are strong chemoattractants for *B. japonicum*, these compounds exhibit no induction of glyceollin resistance and *nod* genes or are only weak *nod* gene inducers. The chalcone isoliquiritigenin and the isoflavone daidzein (as well as genistein on a parallel biosynthetic pathway) are strong inducers of *nod* genes and glyceollin resistance, while the more complex isoflavonoids, glycinol and glyceollin, are less potent symbiotic signals. It is noteworthy that there is no complete correlation between the two inducing activities of the intermediates. While daidzein, genistein, and isoliquiritigenin are both strong *nod* gene inducers and inducers of glyceollin resistance, coumestrol, for example, induces *nod* genes but not glyceollin resistance.

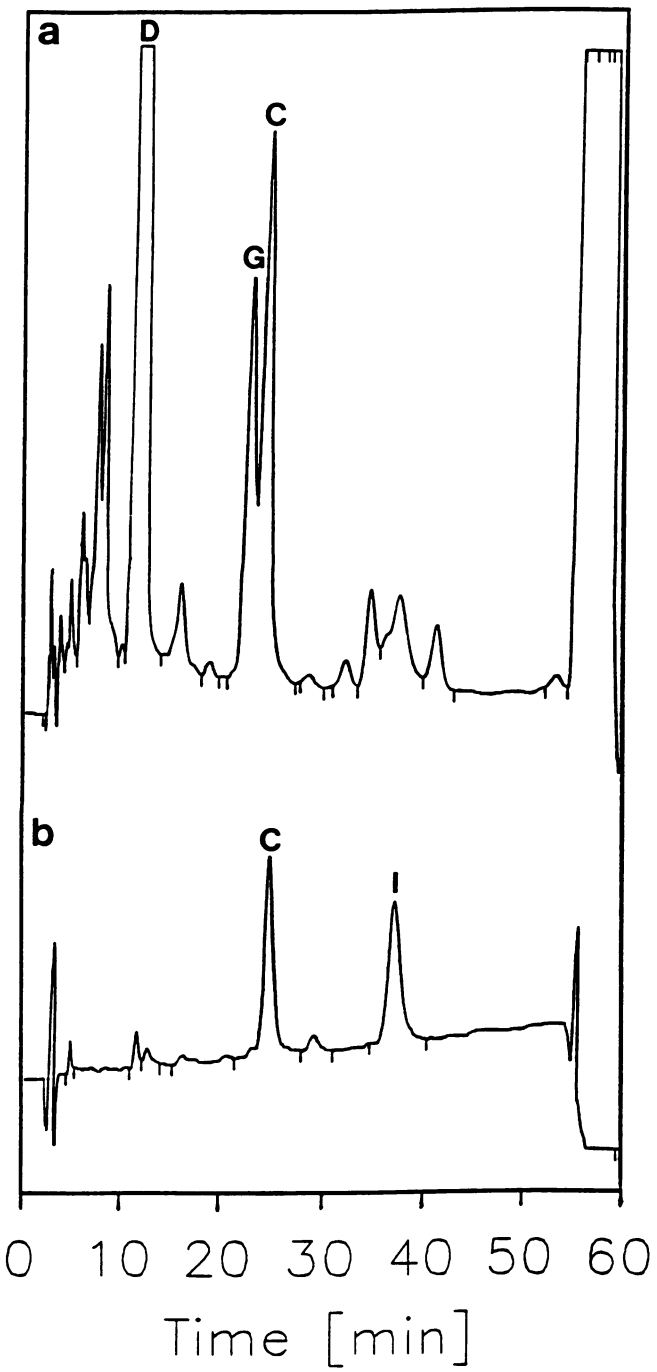

FIG. 2. HPLC profiles of soybean root exudate. UV detection levels at 250 nm (a) and 368 nm (b) are indicated. D, daidzein; G, genistein; C, coumestrol; i, isoliquiritigenin.

DISCUSSION

The results presented here prove that isoliquiritigenin, a component of soybean root exudate, has strong symbiotic activity. *nod* gene induction at very low concentrations and strong induction of glyceollin resistance are the most striking symbiotic features of this compound, which strongly supports a physiological relevance for *B. japonicum* and *G. max* symbiosis. No chemotactic activity of *B. japonicum* in response to isoliquiritigenin was found.

It is important that isoliquiritigenin, a chalcone, is structurally less complex than the strong isoflavonoid *nod* gene inducers for *B. japonicum* described up to now. This finding in connection with those of recently published studies illustrates the broad spectrum of potential *nod* gene inducers for *B. japonicum*. This broad spectrum includes simple phenolic compounds (13) as well as complex flavonoids and isoflavonoids (4, 15). To date, this large diversity of inducing compounds is known only for the broad-host-range rhizobia *Rhizobium* spp. NGR234 and MPIK3030 (2, 11, 16).

The importance of chalcones for *Rhizobium*-legume interaction was recently demonstrated with the *R. meliloti*-alfalfa system (18). In alfalfa root exudate, isoliquiritigenin was missing, but its 2′-methoxy derivative was present. Both chalcones are *nod* gene inducers of *R. meliloti nod* genes. 4,4′-Dihydroxy-2′-methoxychalcone, the stronger inducer of both compounds in *R. meliloti*, is a major component of alfalfa root exudate. This chalcone induced *R. meliloti nod* genes at a concentration 1 order of magnitude lower than luteolin, a flavone (18). Therefore, the *nod* gene-inducing behavior of this alfalfa chalcone is comparable to that of isoliquiritigenin in the soybean root exudate. In both cases, the chalcone seems to be the most effective *nod* gene inducer characterized so far. The reason for the increased *nod* gene-inducing efficiency of isoliquiritigenin in comparison to that of isoflavonoids is unknown. One possible explanation is an enhanced accessibility of the putative site of action in the plasma membrane (30) by isoliquiritigenin through the outer membrane. This speculation is supported by the water solubility of isoliquiritigenin, which is better than that of the isoflavonoids. There is one remarkable difference between the *nod* gene induction behavior of 4,4′-dihydroxy-2′-methoxychalcone in *R. meliloti* and that of isoliquiritigenin in *B. japonicum*. While the alfalfa chalcone produces a maximal *nod* gene induction which is higher than those of the other major inducers, *nod* gene induction by isoliquiritigenin does not reach the maximum induction levels obtained by isoflavonoids. A further similarity between the alfalfa chalcone and isoliquiritigenin in soybean in addition to the high efficiency of *nod* gene induction is apparent. Both chalcones are constituents of the root exudates but are not present in seed exudates (18, 24).

The biological importance of the highly effective isoliquiritigenin, which is present only in low concentrations in the root exudate, is difficult to estimate and has to be evaluated in connection with those of other phenolic compounds with symbiotic activity. As depicted in Fig. 3, effects on *B. japonicum* are not restricted to isoflavonoids. Simple phenolic compounds are chemoattractants but lack the ability to induce glyceollin resistance and *nod* genes or are only weak *nod* gene inducers. The isoflavonoids are not chemoattractants but are strong inducers of glyceollin resistance and of *nod* genes. The different intermediates in the same biosynthetic pathway may be assigned special functions for symbiosis. It remains to be elucidated whether the legume makes use of the multiple functions exhibited by these compounds to manipulate the rhizobial soil population. An active use of this array of substances would be possible only in the case of a spatial regulation of the exudation along the root. Certain local areas of the root would then be characterized by a certain set of exuded compounds and a certain influence on the microsymbiotic partner. First results argue in favor of such a complex spatial regulation of exudation. Gulash et al. (10) observed the attraction of *R. meliloti* cells to localized spots on the root surface of the infectible region of alfalfa

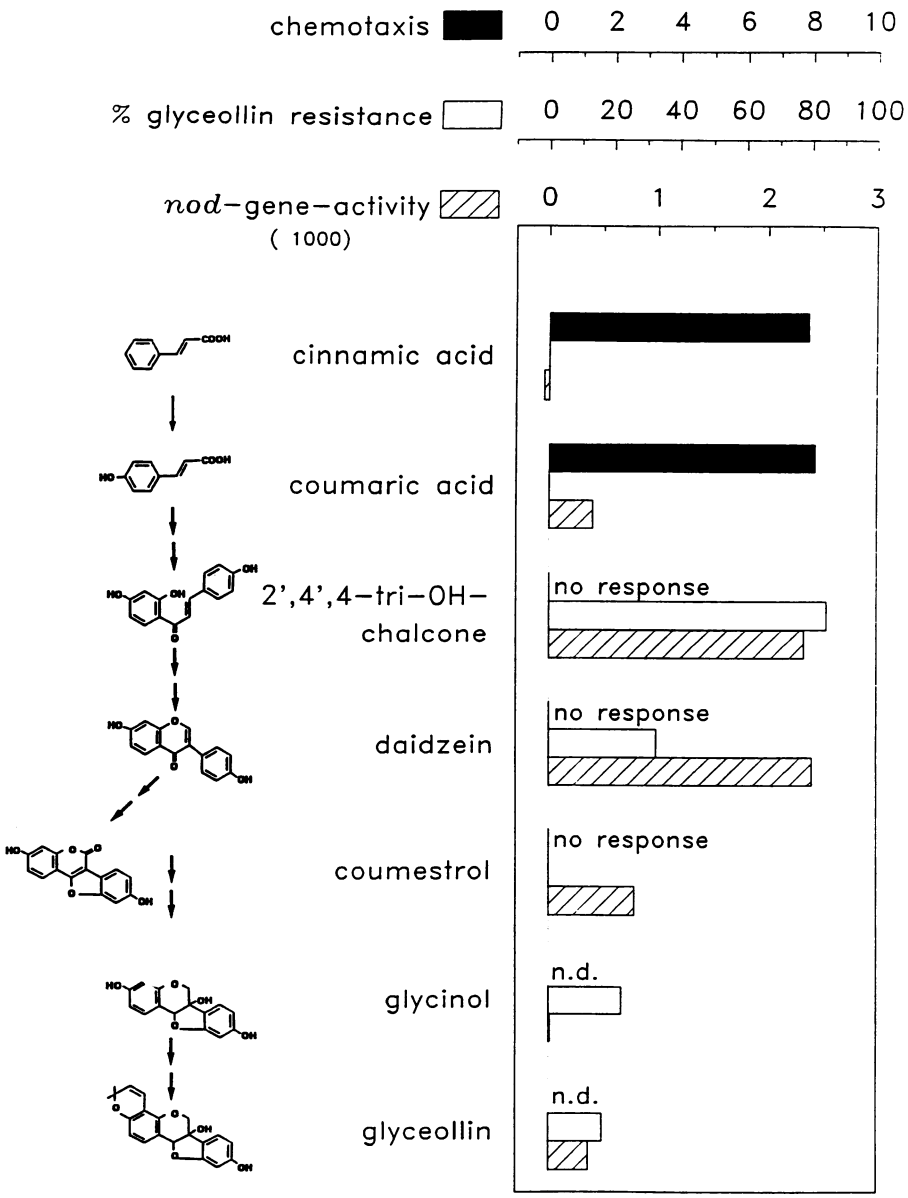

FIG. 3. Differential inductions of symbiotic activities in *B. japonicum* by intermediates of the pterocarpan and the coumestan biosynthetic pathways. Glyceollin resistance data and *nod* gene activation data are from one experiment. Test compounds were applied at a concentration of 10 μM. *nod* gene activity is expressed in β-galactosidase units (in thousands) (according to reference 19). Glyceollin resistance is expressed as the percentage of cells that survived a 3-h incubation in 300 μM glyceollin. The chemotaxis data, except those for isoliquiritigenin, are derived from Kape et al. (13) and are expressed as the chemotactic ratio at the concentration which elicited the chemotactic peak response. n.d., not determined. (Figure 3 is taken from reference 21.)

roots. By using a plate induction assay, Peters and Long (23) demonstrated that the inducers of *R. meliloti nod* genes are exuded from the infectible zone of the root. Graham (8) found distinct differences in the flavonoid-isoflavonoid composition of soybean seedling organs in dependence of the developmental state and the light regime. He elucidated differences in the isoflavonoid contents of individual root segments. Autofluorescence photographs of the soybean root suggest local differences in isoflavonoid concentrations (21). The response of *B. japonicum* to the exuded compounds seems to be dependent on different signal-transducing systems. A chemotactic attraction is the only strong response elicited by simple phenolic acids; therefore, a separate signal transduction system has to be postulated for the chemotactic response. *nod* gene induction and induction of glyceollin resistance have different sets of inducers; e.g., coumestrol, a major component of the root exudate, induces *nod* gene activity but not glyceollin resistance. The induction of glyceollin resistance is also independent of the *nodD* gene product. For these reasons, different mechanisms seem to be inherent to these induction processes. The separate induction processes enable *B. japonicum* to respond differently to individual compounds in the bacterial environment.

The complex spatial regulation of flavonoid exudation into the rhizosphere is not static but highly dynamic. Multiple factors, e.g., the developmental state (23), the presence of

pathogens which may elicit phytoalexin exudation (31), inoculation with rhizobia (26), and abiotic factors (e.g., light [8]) influence the root exudation. Additionally, already exuded substances are subjected to changes by biotic and abiotic factors. Rhizobial as well as legume-derived enzymes modify exuded compounds (8, 12).

When all of its elements are taken together, the rhizosphere seems to be a highly diverse and dynamic space with complex interactions among all inhabiting organisms. The investigation of this complex system is certainly an important step toward the understanding of *Rhizobium* species and legume symbiosis.

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