Nod Factors of *Bradyrhizobium japonicum* and *Rhizobium* sp. NGR234 Induce Flavonoid Accumulation in Soybean Root Exudate

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Flavonoids play an important role as signal molecules in the early stages of symbiosis between legumes and *Bradyrhizobium* or *Rhizobium*. The flavonoid content of root exudates of individual seedlings of *Glycine max* cv. Preston was analyzed by a sensitive method that combines the adsorption of flavonoids on cellulose acetate filter strips and their separation by nano thin-layer chromatography plates, with their identification and quantification by an absorption scanner. *B. japonicum* 110*spc*4, which is able to form nitrogen-fixing nodules with this legume, caused a significant increase in daidzein, coumestrol, and genistein levels in root exudates of infected seedlings. The flavonoid accumulation can be attributed to the presence of suitable rhizobial lipooligosaccharide signals, called Nod factors. This conclusion is based on the following results: 1) *B. japonicum* Δ1240, a Nod⁻ mutant, lacking *nodD*$_1$*D*$_2$*YABCS* and therefore unable to produce Nod factors, failed to induce flavonoid accumulation. 2) Enhanced levels of daidzein, coumestrol, and genistein were also observed after *G. max* seedlings were treated with pure Nod factor of *B. japonicum* in nanomolar concentrations. 3) A similar flavonoid accumulation was induced by a nonsulfated Nod factor of *Rhizobium* sp. NGR234, another soybean-nodulating bacterium. Comparative assays with three Nod factors of NGR234, differing in the substitution of the 2-*O*-methylfucose residue, suggest that in the symbiosis with *G. max* the absence of the sulfate group is essential for the specific induction of flavonoid accumulation. Our data further develop the interaction model involving flavonoids and Nod factors as components of the signal chain between microsymbionts and legume host plants.

Additional keywords: legume symbiosis, nodule formation.

In the symbiosis between legumes and gram-negative soil bacteria belonging to the genera *Rhizobium* and *Bradyrhizobium*, root nodules are formed in which the bacteria actively fix atmospheric nitrogen. The formation of the new plant organ is based on two independent host-specific processes: Initially, rhizobia induce changes in the developmental program of cortical root cells, leading to the formation of nodule meristems (Truchet *et al.* 1991; van Brussel *et al.* 1992). Simultaneously, rhizobia invade the host root through infection threads growing from curled root hairs toward the nodule primordia. During the early stages of the symbiotic interaction, plant defense–like reactions are induced by the invading rhizobia (Djordjevic *et al.* 1987; Schmidt *et al.* 1992). They are thought to be part of the mechanism by which the plant controls the number of infections and thereby regulates nodulation (Vasse *et al.* 1993).

The coordination of these processes involves an intensive exchange of signal molecules between macro- and microsymbiont. Recently, rhizobial signal molecules (Nod factors) capable of eliciting a number of these changes, e.g., nodule meristem formation and root hair curling, have been identified and characterized (Dénarié *et al.* 1992; Fisher and Long 1992). These acylated chitin oligomers are produced by both common and host-specific *nod* genes and are characteristic of each rhizobial strain (Lerouge *et al.* 1990; Spaink *et al.* 1991; Price *et al.* 1992; Sanjuan *et al.* 1992).

As symbiotic plant signals, flavonoids play an important role. In legume seed and root exudates they act as chemoattractants (Aguilar *et al.* 1988; Caetano-Anollés *et al.* 1988), influence the expression of rhizobial *nod* genes (Fisher and Long 1992), and induce the resistance of *B. japonicum* and *R. fredii* against the soybean phytoalexin glyceollin (Parniske *et al.* 1991). Besides their well-known influence on bacteria, flavonoids are currently considered to play a role inside the root during nodule meristem formation (Schmidt *et al.* 1992; Hirsch 1992). The application of certain flavonoids leads to the formation of nodule-like structures on alfalfa roots, an effect that has also been observed with synthetic auxin transport inhibitors (Hirsch *et al.* 1989). Since flavonoids can function as modulators of polar auxin transport (Jacobs and Rubery 1988), it is tempting to speculate that they could locally disturb the auxin-cytokinin balance, leading to the induction of nodule meristems. If this is true, then one would expect alterations of flavonoid content to precede meristem formation. There are reports indicating an influence of symbiotic infection on phenylpropane metabolism. For example, Estabrook and Sengupta-Gopalan (1991) showed that infection of soybean roots with *B. japonicum* leads to enhanced levels of mRNA of symbiosis-specific phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), which are key enzymes in flavonoid production. In 1992, Recourt *et al.* reported enhanced levels of flavonones and chalcones in root exudate of

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Vicia sativa subsp. *nigra* after inoculation with homologous *R. leguminosarum* bv. *viciae*.

Our current working hypothesis is that Nod factor–induced meristem formation involves flavonoids as components of the signal transduction chain. To further examine the role of flavonoids in nodule development, we analyzed the response of soybean roots to *B. japonicum* and different rhizobial Nod factors.

RESULTS

Glycine max root exudates accumulate flavonoids after inoculation with *B. japonicum*.

Previously, we showed that inoculation with *B. japonicum* 110*spc*4 causes a significant increase in glyceollin I levels in root exudates of *Glycine max* (L.) Merr. (Schmidt *et al.* 1992). Here effects of *B. japonicum* on the production of other isoflavonoids in soybean roots were studied with root exudates of single *G. max* seedlings, analyzed for their daidzein, coumestrol, and genistein content. Despite surface sterilization, contamination by seedborne pathogens is inevitable in these assays. In order to exclude the strong flavonoid accumulation effects resulting from defense reactions of contaminated seedlings, experiments were conducted on single seedlings, so that contaminants could easily be identified. In addition, individual measurement would provide better insight into variance of responses and validity of interpretation. This required the establishment of a method sensitive enough to detect changes in the flavonoid content of root exudates of single soybean seedlings. This sensitivity was achieved by 1) selective adsorption of flavonoids on cellulose acetate filter strips, which were coincubated with the seedlings, to facilitate flavonoid extraction from root exudate and minimize contamination, and 2) high-performance thin-layer chromatography (HPTLC), which allows detection of the separated flavonoids in the picomolar range.

The daidzein, coumestrol, and genistein contents of the exudates increased after inoculation with *B. japonicum* 110*spc*4 (Fig. 1). Independent assays revealed that inoculation with that strain always leads to an accumulation of these flavonoids, although to a different extent. While daidzein is quantitatively the major component in exudates from infected and uninfected seedlings, the response in coumestrol and genistein concentration is more pronounced. Despite fluctuations in genistein content, it seems that coumestrol is the predominant component accumulated, since, on average, a more

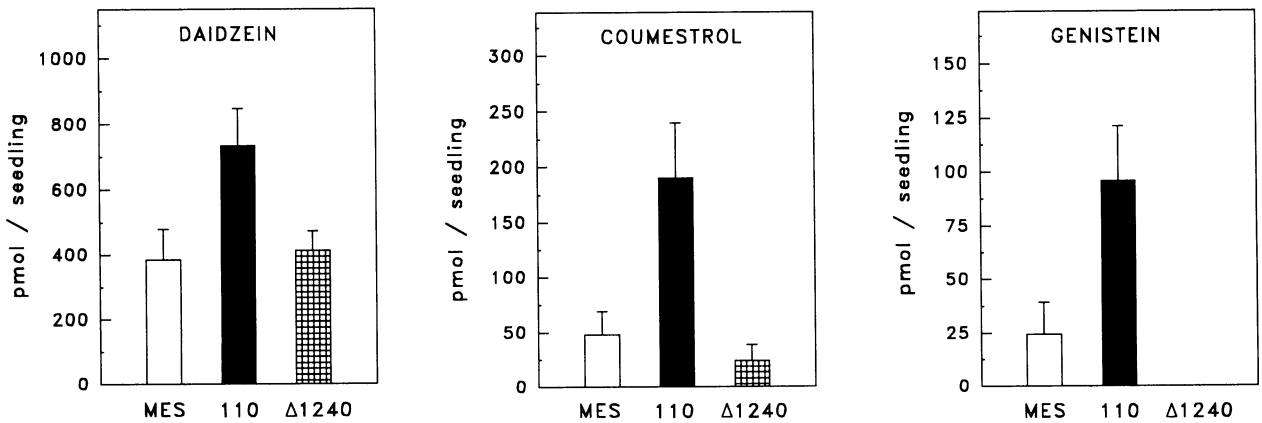

Fig. 1. Amounts of daidzein, coumestrol, and genistein exuded by roots of single seedlings of soybean (*Glycine max* cv. Preston) 18 hr after incubation with morpholinoethanesulfonic acid (MES) buffer (control), *Bradyrhizobium japonicum* 110*spc*4 (10^6 cfu per plant), or the Nod$^-$ mutant *B. japonicum* Δ1240 (10^6 cfu per plant). Genistein exudation after incubation with *B. japonicum* Δ1240 was too low to be measurable. Each value represents the mean of 10 seedlings. Bars indicate SEM values.

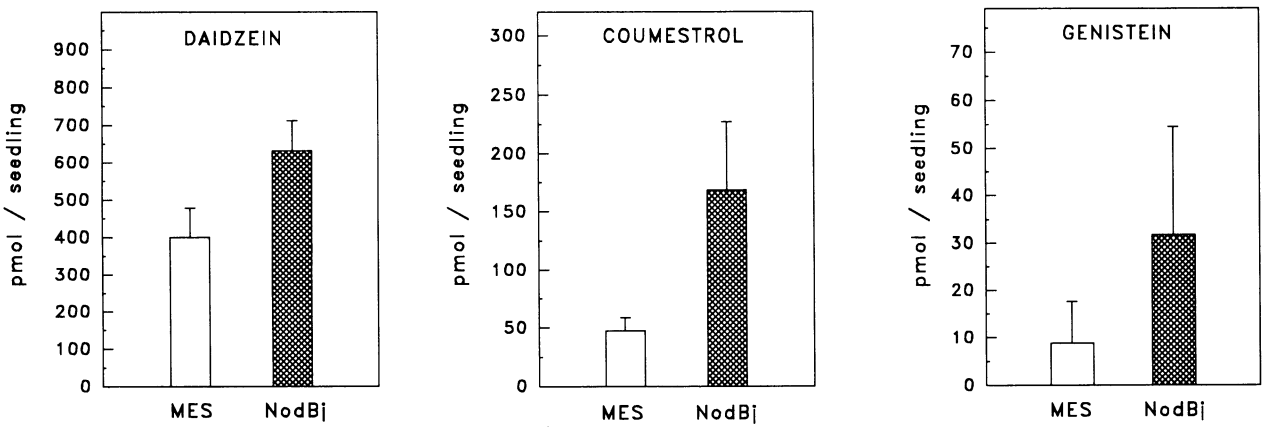

Fig. 2. Amounts of daidzein, coumestrol, and genistein exuded by roots of single seedlings of soybean (*Glycine max* cv. Preston) 18 hr after incubation with morpholinoethanesulfonic acid (MES) buffer (control) or the *Bradyrhizobium japonicum* Nod factor NodBj-V (10 nM). Each value represents the mean of 10 seedlings. Bars indicate SEM values.

constant increase in coumestrol content over that of the control was observed in different assays.

Evidence for Nod factor–induced flavonoid accumulation.

Failure of a B. japonicum Nod⁻ mutant to induce flavonoid accumulation. If flavonoids are a link in the signal chain leading from Nod factor recognition by host root cells to nodule meristem induction, the accumulation of flavonoids might depend on the presence of suitable Nod factors. Accordingly, we inoculated *G. max* seedlings with *B. japonicum* Δ1240, a Nod⁻ mutant, lacking *nodD*$_1$*D*$_2$*YABCS* and therefore unable to produce Nod factors. This mutant failed to induce the flavonoid accumulation observed after wild-type inoculation (Fig. 1).

Flavonoid accumulation after treatment with Nod factors. To test whether pure Nod factor of *B. japonicum* also affects flavonoid production by soybean roots, we inoculated *G. max* seedlings with 10 nM NodBj-V, a Nod factor produced by *B. japonicum*. As shown in Figure 2, NodBj-V induced an accumulation of daidzein, coumestrol, and genistein in root exudate that was fully comparable to that induced by the origin strain, *B. japonicum* 110*spc*4.

In addition to NodBj-V, Nod factors of *Rhizobium* sp. NGR234, another soybean-nodulating bacterium, were tested. This broad-host-range bacterium produces a family of sulfated and nonsulfated Nod factors (Price *et al.* 1992) that have a carbohydrate backbone similar to that of NodBj-V but carry a variety of substituents.

In general, sulfated and nonsulfated Nod factors have been reported to possess different biological activity on legumes. The sulfated Nod factors of *R. meliloti* (Roche *et al.* 1991) and *Rhizobium* sp. NGR234 (Price *et al.* 1992) are more active on *Medicago* than the nonsulfated ones. In contrast, the nonsulfated Nod factors of *R. meliloti* (Roche *et al.* 1991) and of *Rhizobium* sp. NGR234 (Price *et al.* 1992) are more active on *Vicia* than the sulfated ones.

This prompted us to assay sulfated and nonsulfated NodNGR on *G. max*. The two NGR Nod factors that were assayed on *G. max* seedlings differ in the substitution of 2-*O*-methylfucose: in NodNGR-V(Ac), the 2-*O*-methylfucose is 4-*O*-acetylated; in NodNGR-V(S), it is 3-*O*-sulfated. Their flavonoid-enhancing activity was tested with three different dilutions. As shown in Figure 3, the acetylated Nod factor resulted in a clear increase in genistein and coumestrol content, up to 10^{-9} M, and a weaker daidzein response, up to 10^{-10} M. Further assays with 10^{-8} M NodNGR(Ac) showed, on average, even stronger increases in daidzein content relative to that of the control. In contrast, the sulfated Nod factor was more or less ineffective at all concentrations tested. Interestingly, the flavonoid exudation pattern of seedlings treated with the acetylated Nod factor was similar to that induced by *B. japonicum* and by its Nod factor. Both treatments led to significant increases in the amount of coumestrol and genistein, unlike treatment of seedlings with the sulfated factor.

In addition, another nonsulfated NGR Nod factor has been tested on *G. max* (data not shown). The application of 10^{-8} M NodNGR(OH) led to an accumulation of daidzein and coumestrol but failed to raise the genistein content significantly higher than NodNGR(S) did. This finding indicates that with regard to flavonoid accumulation, acetylation as well as lack

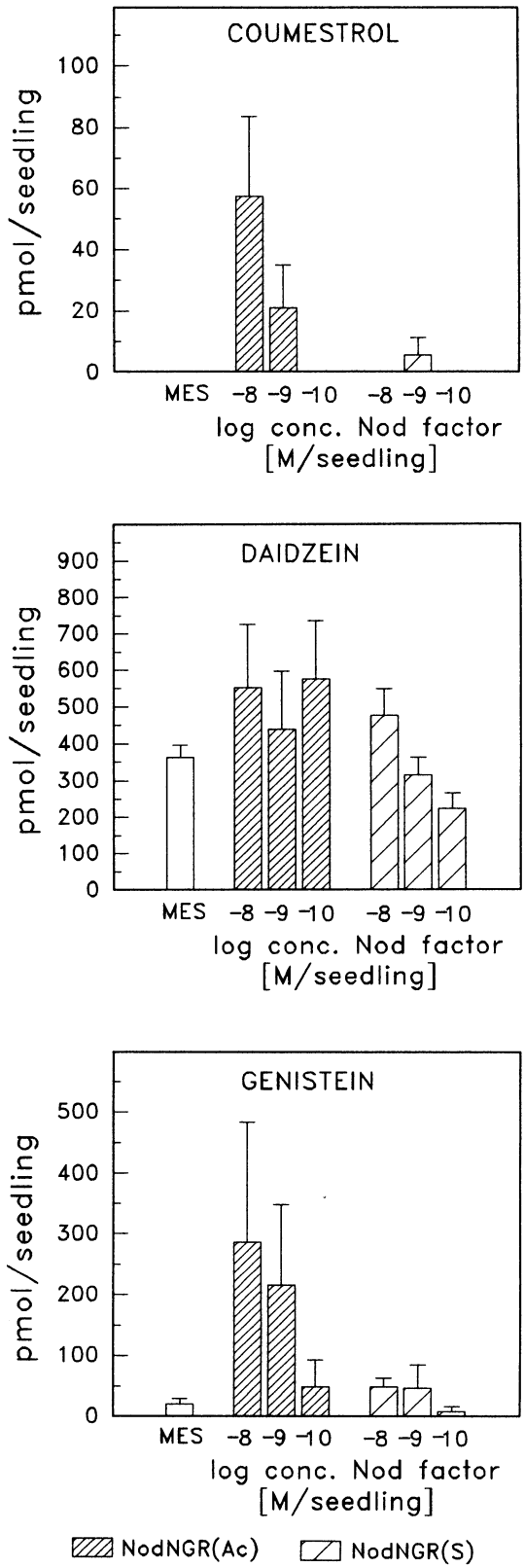

Fig. 3. Amounts of daidzein, coumestrol, and genistein exuded by roots of single seedlings of soybean (*Glycine max* cv. Preston) 18 hr after incubation with morpholinoethanesulfonic acid (MES) buffer (control) or different dilutions (10^{-8}–10^{-10} M) of the Nod factors NodNGR(Ac) and NodNGR(S) from *Rhizobium* sp. NGR234. Each value represents the mean of 10 seedlings. Bars indicate SEM values.

of sulfatation are required for NGR Nod factor activity on *G. max*.

Nod factors of *B. japonicum* and *Rhizobium* sp. NGR234 induce root hair deformation on soybean roots.

Besides their effect on flavonoid accumulation in root exudate, we tested the Nod factors for their biological activity in a root hair deformation assay (Fig. 4). *G. max* seedlings normally respond to *B. japonicum* inoculation with a curling of competent root hairs. The same types of deformation were found on *G. max* exposed to NodBj-V (10 pM–10 nM). Likewise, NodNGR(Ac) (10 pM–10 nM) as well as *Rhizobium* sp. NGR234 produces root hair curling on *G. max*. Interestingly, all three Nod factors of NGR234 produce root hair curling and root hair deformation on *G. max* (data not shown), indicating that the root hair–curling activity of *Rhizobium* sp. NGR234 on *G. max* is independent of the presence or absence of the sulfate group.

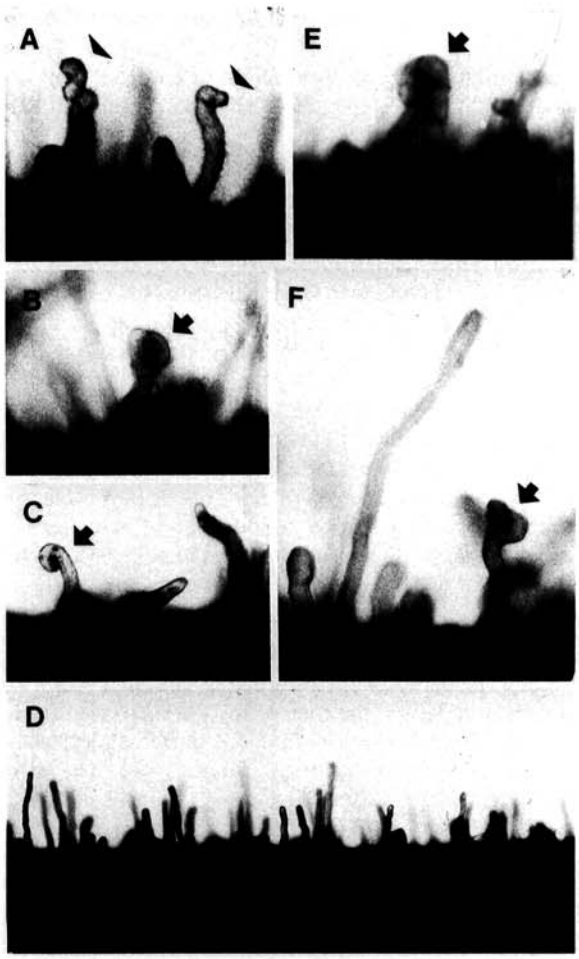

Fig. 4. Light-microscopic examination of the effect of both free-living bacteria and Nod factors of *Bradyrhizobium japonicum* 110*spc*4 and *Rhizobium* sp. NGR234 on deformation (arrowheads) and curling (arrows) of root hairs of *Glycine max* cv. Preston. **A,** Root hair deformation resulting from treatment with 10 pM Nod factor from *B. japonicum*. **B,** Root hair curling after treatment with 1 nM Nod factor from *B. japonicum*. **C,** Root hair curling after treatment with *B. japonicum* 110*spc*4 (5×10^6 cfu). **D,** Section along a root of *G. max* cv. Preston showing undeformed root hairs of a control seedling. **E,** Root hair curling provoked by 10 pM NodNGR factor. **F,** Root hair curling after treatment with *Rhizobium* sp. NGR234 (5×10^6 cfu).

DISCUSSION

Inoculation with *B. japonicum* 110*spc*4 leads to an accumulation of flavonoids in root exudates of its host plant *G. max*. Root exudates of uninfected *G. max* seedlings contain daidzein and small amounts of coumestrol and genistein (D'Arcy-Lameta 1986; Kape 1992). Here we show that in addition to glyceollin, which is produced by soybean roots within the first hours of the symbiotic interaction (Schmidt *et al.* 1992), daidzein, coumestrol, and genistein also accumulate. Using two different approaches, we found that the observed flavonoid accumulation is induced by Nod factors. 1) A *B. japonicum* mutant lacking *nodD*$_1$*D*$_2$*YABCS* failed to affect the flavonoid content of soybean root exudate (Fig. 1). This finding is consistent with the hypothesis that the observed flavonoid accumulation is Nod factor–dependent. Sanjuan *et al.* (1992) showed that strains carrying a Tn*5* insertion in either *nodB* or *nodC* were unable to produce Nod factors. 2) To test whether flavonoid accumulation could also be induced by the addition of isolated Nod factors to the incubation liquid, we exposed seedlings to Nod factors of *B. japonicum* and the soybean-nodulating *Rhizobium* sp. NGR234. As shown in Figures 2 and 3, nanomolar concentrations of NodBj-V and NodNGR(Ac) were sufficient to cause an increase in the flavonoid content of *G. max* root exudate.

Nod factors of NGR234 have a backbone similar to that of NodBj-V. Both are chitin pentamers, N-acylated with a single unsaturated $C_{18:1}$ fatty acid (which in the case of NGR Nod factors may instead be a $C_{16:1}$ fatty acid), carrying a methylfucose substitution on the reducing glucosamine. The presence of the *O*-methylfucose in the lipooligosaccharide backbone seems to be important for activity on *G. max*, since the Nod factors secreted by *R. fredii*, another soybean-nodulating bacterium, are also substituted by a fucose residue (Bec-Ferté *et al.* 1993).

Interestingly, the sulfate group of the NGR factors strongly reduces the flavonoid accumulation activity. Whereas the presence of the sulfate group is required for the effectiveness of the *R. meliloti* factor on *Medicago* (Roche *et al.* 1991), its absence seems to be important for Nod factor activity on *Vicia* (Roche *et al.* 1991) and, with respect to flavonoid accumulation, on *G. max* (Fig. 3). The ability of *Rhizobium* sp. NGR234 to nodulate *G. max* might be connected with its production of a whole family of Nod factors, including different sulfated and nonsulfated Nod factors, active on different hosts (Price *et al.* 1992).

In general, Nod factors are biologically active at pico- to nanomolar concentrations in causing root hair deformation as well as the initiation of meristems from the differentiated cortical cells, leading to the formation of nodule-like structures on the roots of host plants (Truchet *et al.* 1991; Spaink *et al.* 1991). The fact that both meristem induction and flavonoid accumulation are provoked by Nod factors at similar concentrations supports the hypothesis that the processes are physiologically linked. Some flavonoids are described as inhibitors of polar auxin efflux, leading to accumulation of indoleacetic acid in vesicles (Faulkner and Rubery 1992). Since local alterations in the auxin-cytokinin ratio are known to induce cell division, flavonoids may act as growth regulators and initiate cortical cell proliferation (Hirsch *et al.* 1989). The finding of Martinez *et al.* (1993) that several flavonoids,

added in nanomolar concentrations to bean roots, stimulate the formation of nodule-like structures is consistent with the hypothesis that flavonoids possess other important functions in nodule formation besides their role as *nod* gene inducers.

It has also been shown for other legumes that flavonoid accumulation in the rhizosphere is activated by inoculation with the compatible microsymbiont. Van Brussel *et al.* (1990) and Spaink *et al.* (1991) monitored the enhanced flavonoid synthesis by the increased *nod* gene–inducing activity of the root exudate. They showed that inoculation of *V. sativa* subsp. *nigra* roots with the compatible microsymbiont or Nod factors leads to an increased exudation of *nod* gene–inducing components. Our results indicate that the observed flavonoid accumulation is not primarily related to *nod* gene induction, since we found a pronounced accumulation of coumestrol, which is a weak *nod* gene inducer (Kape 1992). Experiments are in progress to further examine the possible role of flavonoids as signal mediators of Nod factors.

Recourt *et al.* (1992) identified eight new flavonoids released by *V. sativa* subsp. *nigra* roots after inoculation with *R. leguminosarum* bv. *viciae*. Dakora *et al.* (1993) found that root exudates of *Medicago sativa* inoculated with *R. meliloti* contained three isoflavonoids that were not found in exudates of uninoculated plants. Cooper and Rao (1992) suggested that rhizobia themselves could be responsible for changes in the flavonoid content of root exudates, either indirectly, through effects of their metabolism on the chemical environment of the rhizosphere, or directly, by degradation of flavones (Rao *et al.* 1991). In our approach this possibility is excluded, since the flavonoid pattern in *G. max* root exudate after inoculation with *B. japonicum* was also induced by its isolated Nod factor.

Coincubation of *G. max* and *B. japonicum* leads to an enhanced expression of specific CHS and PAL genes in the roots (Estabrook *et al.* 1993). In addition, it has been shown by Recourt *et al.* (1992) that inoculation of *V. sativa* subsp. *nigra* with *R. leguminosarum* increases the expression of CHS genes and the enzyme activity of PAL. Since PAL and CHS are key enzymes in flavonoid biosynthesis, it is possible that the observed flavonoid exudation results from a *de novo* synthesis and that the flavonoid content inside the root is also elevated. This conclusion is supported by our results with plants treated with R-(1-amino-2-phenylethyl)phosphonic acid (APEP). After inhibition of flavonoid biosynthesis by the PAL inhibitor APEP, NodBj-V failed to induce the enhanced flavonoid exudation (data not shown), indicating that Nod factors actually induce a *de novo* synthesis of flavonoids associated with their exudation and not an enhanced exudation derived from preexisting pools.

The data presented here further support the model proposed by Schmidt *et al.* (1992), in which flavonoids are part of a more comprehensive signal chain in this symbiosis.

MATERIALS AND METHODS

Growth of seedlings.

Soybean seeds (*G. max* cv. Preston) (Pioneer Hi-Bred International, Johnston, IA) were surface-sterilized for 10 min in 30% H_2O_2, washed 10 times with sterile H_2O, and then soaked for 6 hr in sterile H_2O. Seedlings were germinated on nitrogen-free nutrient agar (Werner *et al.* 1975) for 2 days at 25° C in the dark.

Bacteria and culture conditions.

B. japonicum 110*spc*4 (Regensburger and Hennecke 1983) and the *B. japonicum* mutant Δ1240, lacking *nodD*$_1$*D*$_2$*YABCS* (Göttfert *et al.* 1992), were grown at 28° C on a rotary shaker in succinate minimal medium, a variation of the medium described by Schmidt *et al.* (1992), containing 10 mM succinate as the sole C source.

Nod factors.

Nod factors of *Rhizobium* sp. NGR234 were extracted and purified as described by Price *et al.* (1992); these Nod factors were NodNGR-V(Carb$_2$,NMe,C$_{18:1}$)(MeFuc,Ac), NodNGR-V(Carb$_2$,NMe,C$_{18:1}$)(MeFuc,S), and NodNGR-V(Carb$_2$,NMe,C$_{18:1}$)(MeFuc,OH). The *B. japonicum* 110 Nod factor, NodBj-V(C$_{18:1}$)(MeFuc,OH), was kindly supplied by G. Stacey (Sanjuan *et al.* 1992). Aliquots of the different Nod factors were lyophilized and stored at −20° C. Stock solutions were prepared by dissolving a known weight of component in water/ethanol (50/50). For biological assays they were further diluted with incubation medium.

Inoculation.

Roots of 2-day-old soybean seedlings were transferred to 2.3-ml test tubes. Small tubes were chosen to minimize the dilution of the flavonoids. The tubes contained cellulose acetate filter strips (0.5 × 6.5 cm) (Schleicher & Schüll, Göttingen, FRG) and either morpholinoethanesulfonic acid (MES) buffer (5 mM, pH 6.2), Nod factors, or suspensions of the bacterial strains (see the legends of the figures). The seedlings were incubated under the following growth conditions: 6 hr of light, 10 hr of dark, and then 2 hr of light at 25° C, 13 $W \cdot m^{-2}$, and 75% humidity. Flavonoid content was analyzed after an incubation period of 18 hr, which was just before the space in the small tubes would have become limiting because of the emergence of lateral roots.

Flavonoid identification and quantification.

Exuded flavonoids were adsorbed on the filter strips. After the incubation period, the filter strips were removed from the test tubes, washed twice with MES buffer, dried, and extracted twice with methanol (1.75 and 1 ml). The extracts were gradually evaporated to dryness and resolved in 4.5 µl of methanol. Samples (3 µl) of the concentrated extracts were analyzed for daidzein, coumestrol, and genistein, respectively. Separation of these flavonoids was performed by HPTLC at −18° C on 10 × 10 cm Sil_{60} silica plates (Macherey & Nagel, Düren, FRG) with toluol/ethyl acetate/methanol (70:25:5) as solvent. To enhance resolution, plates and solvent were precooled to −18° C for 1 hr before HPTLC. Spot localization and quantification were performed by scanning plates at 250 nm with a densitometer (CD60, Desaga, Heidelberg, FRG). For spot identification, R_f values and absorption spectra were determined and compared with those of flavonoid standards separated under the same conditions. The concentrations were determined by the use of known standards.

Root hair assay.

Seeds of *G. max* cv. Preston were sterilized in H_2SO_4 for 10 min, rinsed in H_2O, further sterilized in 5% (v/v) H_2O_2 for 5 min, and placed on 0.5% (w/v) agar dissolved in B & D nutrient (Broughton and Dilworth 1971). When the emerging radicle was approximately 1.5–2 cm long (after about 3 days at 26° C), the seedlings were carefully placed in modified Eppendorf tubes contained in test tubes, the bottoms of which had been painted black to exclude light (Price *et al.* 1992). Dilutions of Nod factors prepared from stock solutions dissolved in water/ethanol (50/50) were added to 10 ml of B & D solution (pH 7.0). The Eppendorf tubes were adjusted within the test tubes so that the root tip was just submerged in the B & D solution. Ten tubes were used for each treatment, and the experiments were repeated twice. After 4 days of growth (at a daytime temperature of 30° C and a nighttime temperature of 20° C, with a light phase of 16 hr, including a 1-hr stepped "sunrise" and a 1-hr stepped "sunset," and with illumination at a maximum intensity of 350 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ PAR), the seedling was extracted, and the root excised and stained in filtered 0.1% (w/v) methylene blue dissolved in B & D. The roots were washed in filtered B & D solution and then examined under an inverted microscope.

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