Inoculation with *Azospirillum* Increased Exudation of Rhizobial *nod*-Gene Inducers by Alfalfa Roots

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Azospirillum brasilense caused a significant increase in the *nod*-gene inducing activity of crude alfalfa root exudates when compared to exudates of the noninoculated controls. *nod*-gene inducing activity of exudates from yeast extract-elicited roots was also increased, but to a lesser extent. Fractions separated by HPLC from the exudates of roots treated with *Azospirillum*, yeast extract, and a control showed different *nod*-gene inducing capacities. The profiles of flavonoids extracted from the *Azospirillum*-inoculated roots differed from those of controls, but the difference was less pronounced than was seen in the exudates. The profile of the flavonoids from elicited roots differed greatly from the former two. The data obtained from the flavonoid analyses were in accordance with enzyme activities and mRNA levels of the defense-related proteins phenylalanine ammonia-lyase (PAL), chalcone isomerase (CHI), and isoflavone reductase (IFR); CHI activity and its mRNA were induced in the roots elicited by yeast extract, whereas the activities of PAL and CHI, and mRNA levels of PAL, CHI, and IFR were not induced by *Azospirillum*.

Additional keyword: Medicago sativa.

Free-living nitrogen-fixing azospirilla exert beneficial effects on plant growth and increase yields of many crops of agronomic importance (Okon and Labandera-Gonzalez 1994). The interaction of *Azospirillum* with the plant generally is not host specific (Fallik et al. 1994). The increase in yield is mainly attributed to an improvement in root development, increase in the rate of water and mineral uptake, and, to a lesser extent, to biological N₂ fixation (Okon and Kapulnik 1986). It is assumed that *Azospirillum* affects plants mainly by the production of plant growth-promoting substances, which leads to enhanced root development and, thus, to general plant growth promotion (Fallik et al. 1994).

Bacteria of the genus *Rhizobium* interact with leguminous plants in a host-specific manner and form N₂-fixing root nodules (Long 1989). Alfalfa (*Medicago sativa*) roots are nodulated by *Rhizobium meliloti* (Brockwell and Hely 1966). Successful establishment of the symbiotic interaction involves chemotaxis of the bacteria towards the roots, root colonization, root hair deformation, infection thread formation, and

rapid division of root cortex cells (Long 1989). Early events of alfalfa nodule formation require expression of nodulation genes including *nodDABC* on the megaplasmid of *R. meliloti* (Dudley et al. 1987; Kondorosi et al. 1984). Signal molecules of plant origin initiate the symbiotic interaction, and in alfalfa those molecules have been identified as flavonoids and betaines (Phillips et al. 1994). Inoculation with *Rhizobium* increases secretion of *nod*-gene inducing flavonoids (Dakora et al. 1993), and in alfalfa, as in other legumes, antimicrobial phytoalexins and the *nod*-gene inducers are biosynthetically related (Dixon et al. 1992).

Enhanced nodulation by combined inoculation with *Azospirillum* and *Rhizobium* has been reported for several legumes (Volpin and Kapulnik 1994). In alfalfa roots, dual inoculation caused an increase in main root nodule count as well as early nodulation (Itzigsohn et al. 1993). It has been hypothesized that the beneficial effect *Azospirillum* exerts on nodulation is due to changes in root morphology caused by *Azospirillum*, such as enhanced root hair formation, and thus the creation of more potential infection sites for *Rhizobium* (Volpin and Kapulnik 1994). Additional factors may, however, be involved. In light of the fact that the presence of *Rhizobium* enhances secretion of *nod*-gene-inducing flavonoids (Dakora et al. 1993), and that many microorganisms elicit changes in flavonoid metabolism (Dixon et al. 1992), we suggest that part of the effect of *Azospirillum* on nodulation may be through such changes in root exudation. The objective of this research was to study the effect of *Azospirillum* on the secretion of *nod*-gene inducing flavonoids. Prior to the onset of changes in flavonoid secretion, flavonoids, enzyme activities of phenylalanine ammmonia-lyase (PAL) and chalcone isomerase (CHI), and steady state mRNA levels of PAL, CHI, and isoflavone reductase (IFR) of the roots were observed in an attempt to characterize the mechanism by which *Azospirillum* affected root exudation. In addition, yeast extract was used to induce a nonspecific defense response (Dixon et al. 1992) to test whether any changes associated with *Azospirillum* could be attributed to a general increase in flavonoid metabolites.

RESULTS

nod-Gene inducing activities of root exudates.

Inoculation with *Azospirillum* increased *nod*-gene inducing activity of crude alfalfa root exudates by a factor of 23 and 14, 48, and 96 h after inoculation, respectively (Fig. 1), as com-

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pared with the controls. Crude root exudates from plants elicited with autoclaved yeast extract also enhanced nod-gene activity, but to a lesser extent (50 to 70% of the effect of Azospirillum) (Fig. 1).

When the root exudates were separated into fractions by HPLC, fractions of exudates from Azospirillum inoculated plants eluting between 16 and 20 min showed higher nod-gene inducing activity than the fractions from the controls (Figs. 2D, E). Comparable eluting fractions of exudates from plants elicited with yeast extract did not show any nod-gene inducing

Fig. 1. Relative induction of nod genes by alfalfa root exudates. Samples were collected at 48 and 96 h after inoculation of 4-day-old plants. Transcription of nodABC-lacZ on pRmM57 in Rhizobium meliloti 1021 was measured as β-galactosidase activity. Relative nod-inducing activity represents the exudate dilution equivalent to β-galactosidase activity induced by 0.2 µM of luteolin, per 1g of root (fresh weight). Each value represents the mean ± SE of two to three replicates (400 plants each).

activity (Fig. 2F). Comparison of UV absorption spectra of the eluting compounds revealed almost no similarity among the compounds from the different treatments. Spectra of fractions eluting between 24 and 40 min showed a higher degree of similarity, but significant quantitative differences between the treatments were evident (Fig. 2A–C). Only fractions from the yeast extract-treated plants eluting between 44 to 46 min showed nod-gene significant inducing activity (Fig. 2F), and the spectra of these compounds were very different from those of the Azospirillum-treated plants or the controls (results not shown).
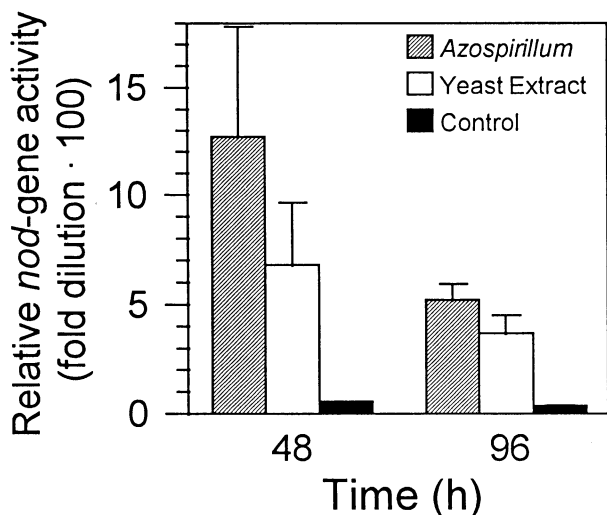
Flavonoid content of roots

HPLC chromatograms of MeOH extracts from alfalfa roots revealed several metabolic differences among the treatments 8 to 24 h after inoculation (Fig. 3). Peaks number 1, 2, 3, and 4 have previously been identified as two formononetin-7-O-glycosides, medicarpin-3-O-glycoside and formononetin, respectively (Volpin et. al. 1995). Peak number 5 had UV-visible absorbance spectra identical to that of medicarpin, and cochromatographed with an authentic standard of this compound.

A minor decrease in levels of formononetin-7-O-glycosides was evident in the Azospirillum-treated roots between 16 and 24 h after inoculation. At this time, a minor increase in the yeast extract-treated roots was observed, as compared with the controls (Fig. 4A). Formononetin levels were lower in the Azospirillum-treated roots than in the controls between 12 and 24 h after inoculation, except at 20 h when it was significantly higher (Fig. 4B). Formononetin was below detectable levels in the yeast extract-treated roots. No differences could be observed in medicarpin-3-O-glycoside levels between roots treated with Azospirillum and untreated controls, but in the yeast extract-treated roots the amount of medicarpin-3-O-glycoside was increased two- to threefold (Fig. 4C). The amount of medicarpin was below detectable levels in roots treated with Azospirillum and in the untreated controls, except
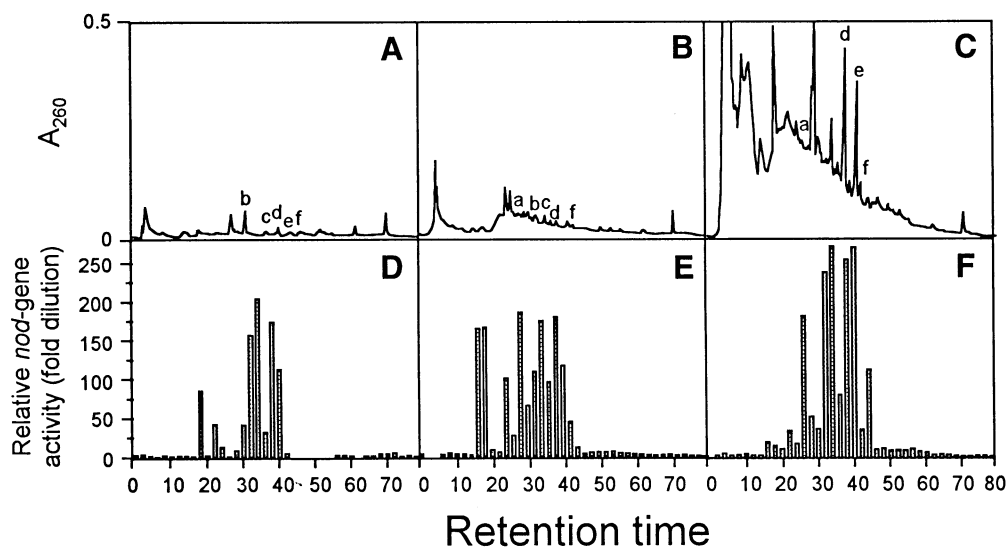

Fig. 2. HPLC characteristics (A_{260}) (A–C) and nod-gene induction assays (D–F) of the flavonoid fraction from root exudates (100 ml) of alfalfa (Medicago sativa) plants collected 48 h after inoculation of 4-day-old plants. Untreated controls (A,D), roots inoculated with Azospirillum brasilense cd (b,e), and roots elicited with yeast extract (3 mg ml⁻¹) (c,f). Relative nod-inducing activity represents the exudate dilution equivalent to β-galactosidase activity induced by 0.2 µM of luteolin, per 1 g of root (fresh weight). Peaks marked with the same letters had identical spectra and retention times.

at 8 h after inoculation when the roots treated with *Azospirillum* contained 0.03 mg/g fresh weight (Fig. 4D). The roots treated with yeast extract contained between 0.06 to 0.08 mg/g fresh weight, except at 12 h after inoculation when the amount reached about 0.11 mg/g fresh weight (Fig. 4D).
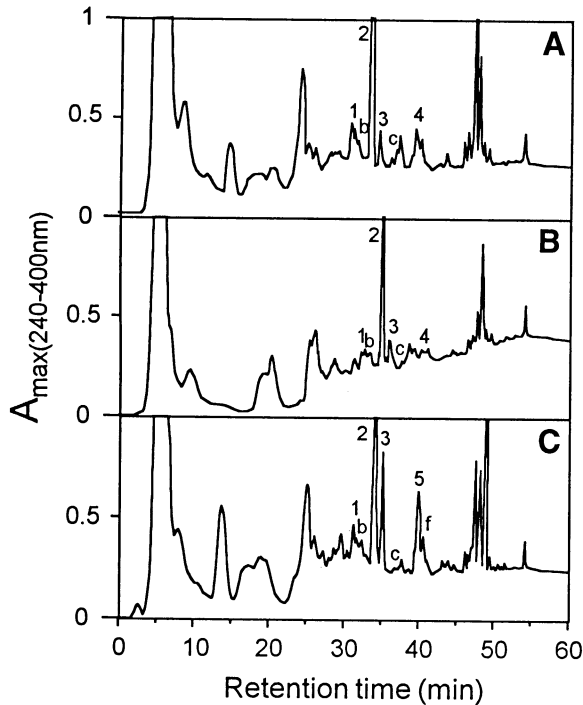

Fig. 3. HPLC characteristics ($A_{max(240-400nm)}$) of extracts from 4-day-old alfalfa roots, 12 h after inoculation. Untreated controls (**A**), roots inoculated with *Azospirillum brasilense* cd (10^6 to 10^7 CFU ml^{-1}) (**B**), roots elicited with yeast extract (3 mg ml^{-1}) (**C**). Peaks are identified here as formononetin-7-*O*-glycosides (1-2), medicarpin-3-*O*-glycoside (3), formononetin (4), and medicarpin (5). Peaks marked with letters had spectra and retention times similar to those marked with identical letters in Figure 2.
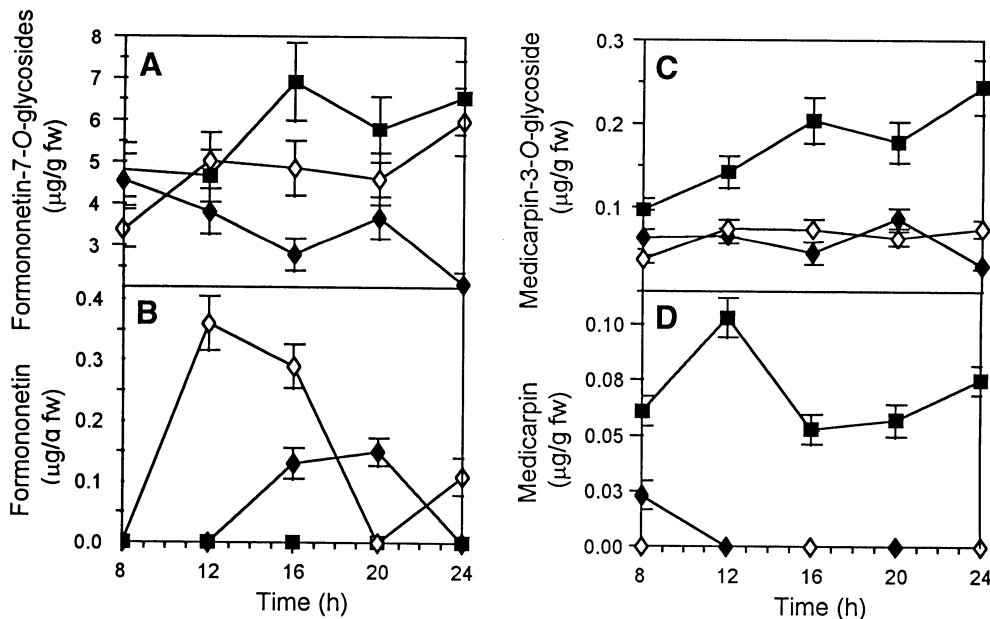
Enzyme activities.

PAL and CHI activities in crude extracts of roots inoculated with *Azospirillum* did not differ from those of the untreated controls. PAL activities of the yeast extract-treated roots were higher than those of the controls throughout the experiment, but the differences were not significant at any given point. CHI activities were significantly higher in the yeast extract-treated roots between 12 and 24 h after inoculation (Fig. 5) than in controls.

RNA analysis.

When total RNA was extracted from the alfalfa roots, relative amounts of steady-state CHI mRNA in roots elicited with yeast extract were shown to have increased significantly between 8 to 24 h after inoculation (Fig. 6). The same parameter showed little change in *Azospirillum* treated and nontreated control roots. Over the same period, no differences could be observed in the relative amounts of PAL or IFR mRNA's among the different treatments.

DISCUSSION

Results from this study show that *Azospirillum* causes a 14- to 23-fold increase in the *nod*-gene inducing activity of crude alfalfa root exudates (Fig. 1). *nod*-gene inducing activity of exudates from yeast extract-elicited roots was also increased, but to a lesser degree (about 10-fold) (Fig. 1). In comparison, inoculating alfalfa roots with *Rhizobium meliloti* has been reported to show only a threefold increase in the *nod*-gene inducing activity of the crude root exudates (Dakora et al. 1993).

Flavonoid profiles of exudates from controls and *Azospirillum* differed more qualitatively than quantitatively (Figs. 2A, B), while the profile from the elicited roots was very distinct, quantitatively and qualitatively from both controls and *Azospirillum*-treated roots (Fig. 2A–C). *nod*-gene inducing

Fig. 4. Flavonoids extracted from alfalfa roots inoculated with *Azospirillum brasilense* (filled diamond), untreated controls (open diamond) or elicited with yeast extract (■). Formononetin-7-*O*-glycosides (**A**), Formononetin (**B**), medicarpin-3-*O*-glycoside (**C**), or medicarpin (**D**). Each value represents the mean ± SE of three replicates (400 plants each).

capacities of the fractions from the three treatments were very different from each other, especially during the first 25 min. Both defense-related and *nod*-gene inducing flavonoids are products of the phenylpropanoid pathway, although the compounds derive from different branches. Several flavonoids secreted by alfalfa roots which induce *nod*-gene activity have been identified, such as 4´,4-dihydroxy-2´-methoxychalcone, 7,4´-dihydroxyflavone, and liquiritigenin (Phillips 1992).

The compounds specific for the *Azospirillum*-plant interaction were not identified, but none of them have spectra similar to these compounds.

When integrating the *nod*-gene inducing activities of the different fractions from the various treatments, the total shows fewer differences than those found in the activity of the crude exudates. This could be attributed either to differences in the competitive interactions of the compounds (we did not check for inhibitors) or differences caused by the presence of hydrophilic *nod*-gene inducing compounds, such as trigonelline or stachydrine (Phillips et al. 1992), lost during the initial purification process of the exudates for HPLC.

In an attempt to analyze the mechanism by which *Azospirillum* influences exudation of *nod*-gene inducers, alfalfa roots were examined for changes in flavonoid metabolism prior to the observed changes of exudation. The profiles of flavonoids exuded from *Azospirillum*-inoculated roots differed from those extracted from the noninoculated controls, but the difference was less distinct than among the exudates. Several of the compounds found in the exudates from *Azospirillum*-treated roots were below detectable levels in the root extracts. The profile of the extracted flavonoids from the yeast extract-elicited roots was greatly different from both that of the *Azospirillum*-inoculated roots and that of the noninoculated controls. Some differences were observed in the quantities of formononetin-7-*O*-glycoside and formononetin found in *Azospirillum* and control, but levels of medicarpin-3-*O*-glycoside were similar, and the amount of medicarpin was below detectable levels in both treatments. Elicited treated roots showed a typical defense response (Dalkin et al. 1990) with accumulation of medicarpin-3-*O*-glycoside and medicarpin.

The data obtained from the flavonoid analysis was strengthened by enzyme activities of PAL and CHI and mRNA levels of PAL, CHI, and IFR. *Azospirillum* did not induce any of these defense-related proteins, neither at the enzyme activity level nor at the mRNA level, whereas CHI activity and mRNA were induced in the roots elicited by yeast extract. In this context, it is interesting to note the different results obtained here as compared with alfalfa cell suspensions elicited with yeast extract. In cell suspensions both PAL, CHI, and IFR were induced (Dixon et al. 1992). In the whole root system, base levels of PAL and IFR may already be high. This, however, creates a problem in the interpretation of the results, as yeast extract did not induce either PAL or IFR mRNA although it can potentially do so. We cannot, therefore, eliminate the possibility that *Azospirillum* could also have this ability. We do not think that this is the case, as flavonoid profiles from *Azospirillum*-treated roots did not show the typical signs of a defense response displayed by the elicited roots. These findings are consistent with the observation that *Azospirillum* did not cause a defense response of maize roots (Fallik et al. 1988).

The manner in which *Azospirillum* affects flavonoid secretion is not clear. It does not seem to cause an increase in flavonoid production in the roots; if anything, a minor decrease could be detected (Figs. 3 and 4). However, qualitative changes in the metabolism are clear. Flavonoid content of the
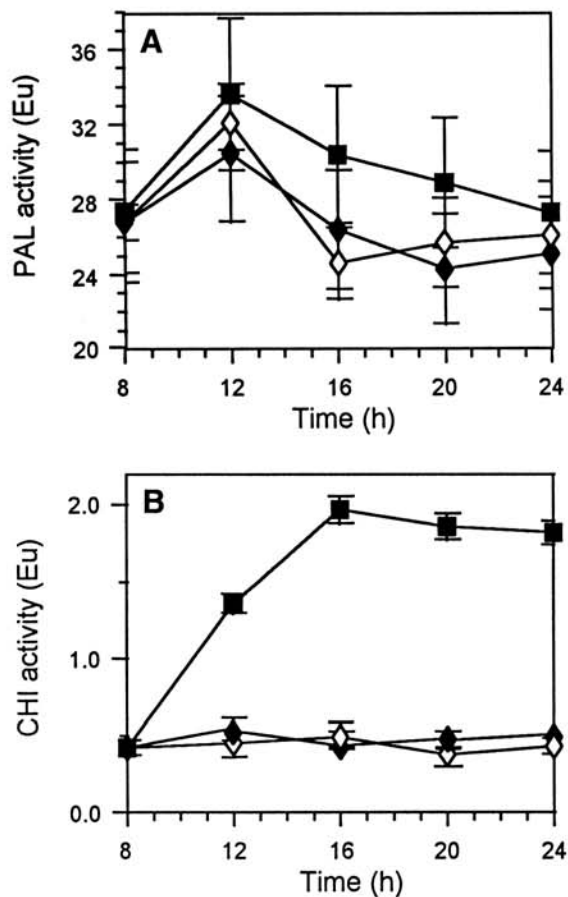

Fig. 5. Phenylalanine ammonia-lyase (PAL) (**A**) and chalcone isomerase (CHI) (**B**) enzyme activities of alfalfa roots inoculated with *Azospirillum brasilense* (filled diamond), untreated controls (open diamond), or elicited with yeast extract (■). Each value represents the mean ± SE of three replicates (400 plants each).
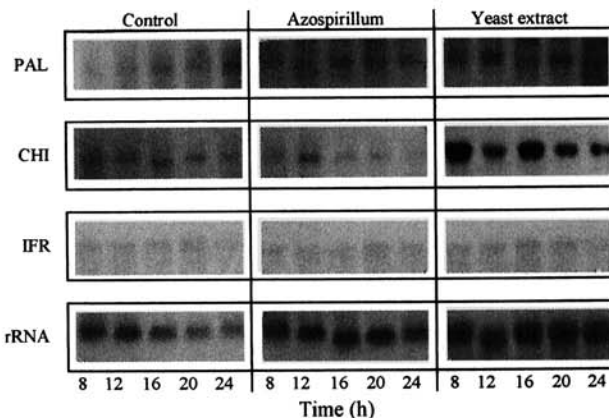

Fig. 6. Changes in mRNA for phenylalanine ammonia-lyase (PAL), chalcone isomerase (CHI), isoflavone reductase (IFR), and rRNA of alfalfa roots inoculated with *Azospirillum brasilense*, untreated controls, or elicited with yeast extract.

roots is developmentally regulated (Tiller et al. 1994), and *Azospirillum* changes root morphology (Fallik et al. 1994). Morphological changes in our experiments were not obvious; however, increased root hair formation has been reported for tomato seedlings inoculated with *Azospirillum* within a 24 h period (Hadas and Okon 1987). *Azospirillum* decreased the amount of conjugated IAA, IBA, and gibberelin and increases the amount of the free compounds in maize (Fallik et al. 1994). Auxin can inhibit flavonoid synthesis (Ozeki et al. 1987; Rengel and Kordan 1987). Its effect, if any, on the balance between the different branches of the pathway is not known.

We conclude that *Azospirillum* increases *nod*-gene activity. When comparing the results with those obtained by nonspecific elicitation, it is evident that *Azospirillum* caused specific changes in flavonoid metabolism, which are not part of a defense response.

MATERIALS AND METHODS

Plant growth and inoculation with *Azospirillum brasilense*.

Alfalfa (*Medicago sativa* L. 'Gilboa') seeds (1 g, about 400 seeds) were surface sterilized for 7 min in 70% EtOH, rinsed with sterile water, and imbibed in aerated water for 4 h. Imbibing solution was changed 3 to 4 times to remove seed-derived compounds. The seeds were then placed on a sterile cheesecloth-covered steel screen over 350 ml of sterile aerated 10-fold diluted Jensen nutrient solution (Vincent 1970) in a 400-ml plastic container. A sterile clear plastic cover was positioned over the container and roots developed into the nutrient solution (Maxwell 1989). Containers were maintained under an irradiance of 40 μE m^{-2} s^{-1}, 16/8 h light/dark, and 25/20°C. Nutrient solutions were changed every 48 h. Treatments were performed on 4-day-old plants. *Azospirillum brasilense* strain cd was grown overnight in liquid malate minimal medium (Okon et al. 1976) and washed three times in sterile tap water by centrifugation (1,000 × g, 5 min). The rinsed bacteria were inoculated into the nutrient solution at a final concentration of 10^6–10^7 CFU ml^{-1}. Elicited controls were obtained by applying autoclaved yeast extract to the growth container at a final concentration of 3 mg ml^{-1} (Jorrin and Dixon 1990). Untreated controls were growth containers supplied with an equal amount of nutrient solution.

Solutions containing root exudates were changed and freeze-dried 48 and 96 h after treatment. Root samples were collected every 4 h, 8 to 24 h after treatment, immediately frozen in liquid N$_2$ and kept at −80°C until assayed.

Assays were conducted in two to three replicates, each replicate containing roots from 400 seedlings. Results are presented with SE values. All experiments were repeated at least twice.

Isoflavonoid isolation.

Exudates. Freeze-dried root exudates were dissolved in 15 ml of HPLC-grade water (Merck, Germany), centrifuged (20 min, 6,000 × g) and passed through 0.2 μm polycarbonate filters (Schleicher & Schuell, Germany). The supernatant was adsorbed into C18 Maxi-Clean Cartridges (Alltech Associates Inc., Deerfield, IL). Flavonoids were eluted with 5 ml of acetone, concentrated to a final volume of 3 ml with a N$_2$ gas stream at 25°C (Maxwell et al. 1989). The flavonoids were

then divided into 1-ml samples which were dried under vacuum. For HPLC samples were dissolved in 120 μl of 50% MeOH. Aliquots (100 μl) were loaded onto a Waters HPLC system (Millipore Corp., Milford, MA) fitted with a 250 × 4 mm LiChrospher 100 RP-18 column and eluted at 0.5 from 0 to 10 min with a linear gradient from 69:30:1 (v/v/v) water/MeOH/acetic acid to 49:50:1 (v/v/v) water/MeOH/acetic acid, from 10 to 60 min with a linear gradient to 99:1 (v/v) MeOH/acetic acid, and the analysis continued isocratically at that concentration for another 20 min. Eluting compounds were monitored with a Waters 990 photodiode array detector. Eluting fractions were collected on a fraction collector (FRAC-100, Pharmacia) every 2 min and dried under vacuum.

Root extracts. Frozen, ground root tissue (1 g) was thawed in 6 ml of 100% MeOH, extracted for 4 h at 4°C and centrifuged at 10,000 × g, 4°C for 25 min. Supernatant was partitioned twice against hexane, dried under vacuum, and dissolved in 200 μl of 50% MeOH. Aliquots (100 μl) were loaded onto a Waters HPLC system (Millipore Corp., Milford, MA) fitted with a 250 × 4 mm LiChrospher 100 RP-18 column and eluted at 0.5 ml·min^{-1} from 0 to 65 min with a linear gradient from 69:30:1 (v/v/v) water/MeOH/acetic acid to 99:1 (v/v) MeOH/acetic acid. The analysis continued isocratically at that concentration for another 10 min. Eluting compounds were monitored with a Waters 990 photodiode array detector.

nod-inducing capacity.

nod-inducing capacity of root exudates and HPLC fractions was assayed as β-galactosidase activity from transcription of the *nodC-lacZ* fusion on plasmid pRmM57 in *Rhizobium meliloti* '1021' (Mulligan and Long 1985), obtained from S. R. Long (Stanford University). β-Galactosidase assay was carried out according to Miller (1972). *nod*-inducing activity was assayed on dilution series of the root exudates and relative *nod*-inducing activity represents the exudate dilution equivalent to β-galactosidase activity induced by 0.2 mM of luteolin, per 1 g of root; 0.2 mM luteolin was within the range of luteolin concentrations causing a linear increase in *nod*-inducing activity, and this concentration would typically induce an activity of about 490 U.

Enzyme extraction and assay.

All enzyme assays were performed with crude extracts.

PAL was assayed by measuring the conversion of L-phenylalanine to cinnamic acid at 270 nm (Lamb et al. 1979). One Eu is defined as the formation of 1 μM cinnamic acid per s per kg of enzyme.

CHI was assayed by measuring the rate of disappearance of the chalcone isoliquiritigenin (synthesized according to Nadkarni and Wheeler (1938)) at 400 nm (Dixon and Bendall 1978). One Eu is defined as the disappearance of 1 mM isoliquiritigenin per s per kg of enzyme.

RNA analysis.

Total RNA was extracted from 0.3 g of root tissue. The tissue was ground to fine powder with liquid nitrogen using mortar and pestle. The powder was then thawed and further ground in 820 ml of RNA grinding buffer (8 M guanidium·HCl, 20 mM Mes, 20 mM EDTA, 50 mM β-mercaptoethanol) and 650 ml of acid phenol/chloroform/isoamyl alcohol (25:24:1 ratio). The mixture was trans-

ferred to a 2-ml Eppendorf tube, shaken vigorously for 15 s and incubated at room temperature (25°C) for 2 to 20 min. Phase separation was done by centrifugation for 15 min. (14,000 rpm) in a microfuge at room temperature. Total RNA was precipitated from the upper phase by adding 1 volume of cold (–20°C) isopropanol, incubating at room temperature for 10 min. and centrifuging for 20 min (12,500 rpm) in a microfuge at 4°C. The pellet was washed twice with 75% ethanol and air dried. The RNA was resuspended in 50 ml of H_2O by incubation at 60°C for 10 min and stored at –80°C until use.

RNA samples (5 to 10 mg) were separated by electrophoresis on formaldehyde gels and blotted on nylon membranes (Nytran, Dassel, Germany) (Sambrook et al. 1989). RNA blots were hybridized with radioactively labeled (^{32}P-dCTP) cDNA probes from pAPAL1 (Gowri et al. 1991), pIFR alf1 (Paiva et al. 1991) and a 943-bp fragment of an alfalfa clone for chalcone isomerase (McKhann and Hirsch 1994). A 1,150-bp fragment of tomato ribosomal RNA gene cloned in pUC8 (provided by E. Lifschitz, Haifa University, Haifa, Israel) was used as the control for loading. All probes were prepared using a random primer DNA labeling kit (Boehringer Mannheim, Germany). Blots were hybridized 14 h at 60°C in 5× Denhardt, 5× SSPE, 1% SDS. Membranes were washed twice, 10 min per wash, in 2× SSC, 1% SDS at room temperature, and then twice, 15 min per wash, in 0.1× SSC, 1% SDS at 60°C. Radiographs were scanned and analyzed by ImageQuant (Molecular Dynamics Personal Densitometer, Sunnyvale, CA). Transcript levels were normalized with respect to the rRNA.

ACKNOWLEDGMENTS

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