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Chemotaxis and *nod* Gene Activity of *Bradyrhizobium japonicum* in Response to Hydroxycinnamic Acids and Isoflavonoids

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For *Bradyrhizobium japonicum*, the chemotactic and the *nod* gene-inducing effects of hydroxycinnamic acids and two of their derivatives were compared with those of isoflavonoids. Only the hydroxycinnamic acids were strong chemoattractants, while the other substances tested were chemotactically inactive. Besides the known *nod* gene induction by isoflavonoids, a weak *nod* gene induction by coniferyl alcohol, chlorogenic acid, and ferulic acid was found.

Bradyrhizobium japonicum, which forms nitrogen-fixing root nodules in symbiosis with *Glycine max*, *Macroptilium atropurpureum*, and some other legume species (10), is a motile soil bacterium with a single subpolar flagellum (30).

Electron micrographs of this flagellum (Fig. 1) clearly demonstrate the typical cross-hatched pattern of a complex flagellum, which has already been described for a number of other soil bacteria (17), but not yet for *B. japonicum*.

B. japonicum responds chemotactically to gradients of certain chemicals (24). Chemotaxis in general has recently been reviewed (1, 7). The importance of rhizobial motility and chemotaxis for the *Rhizobium*-legume symbiosis has been the point of interest of a number of studies. Focusing on a possible importance of chemotaxis for specificity in partner recognition, Gaworzewska and Carlile (12) investigated the chemotactic effects of crude and fractionated root exudates of host and nonhost plants on different *Rhizobium* species. No direct correlation between chemotactic effect and specific nodulation was found.

Flavonoid compounds have been identified as *nod* gene inducers in several plant-*Rhizobium* systems (11, 25, 28). Recent work elucidated the chemotactic effect of these *nod* gene-inducing substances. Caetano-Anollés et al. (8) found for *Rhizobium meliloti* a close correlation between *nod* gene-inducing abilities and the chemotactic effects of flavonoids. The chemotactic response of *R. meliloti* towards flavonoids was strictly dependent on the presence of intact *nod* genes. For *Rhizobium leguminosarum* biovar *viciae*, this correlation between intact *nod* genes and chemotaxis was far less pronounced (3). The results of Aguilar et al. (2) did not indicate a necessary correlation of the chemotaxis of *R. leguminosarum* biovar phaseoli towards flavonoids with the *nod* gene induction by these substances. Still, an involvement of rhizobial chemotaxis towards flavonoids in the initiation of root nodule formation was suggested as well as a possible effect of chemotaxis on the host range. Results similar to those obtained by Caetano-Anollés et al. (8) for *R. meliloti* were found for *Agrobacterium tumefaciens* (4). For this organism, a strong correlation between *vir* gene induction and a Ti plasmid requirement for chemotaxis was established. Acetosyringone, sinapic acid, and a number of other phenolic substances acted as *vir* inducers and chemoattractants in strains harboring the Ti plasmid but not in plasmid-cured strains.

In the present work, the chemotactic behavior of *B. japonicum* was studied. The work focuses on two classes of phenolic compounds, the isoflavonoids and the hydroxycinnamic acids. The isoflavonoids tested in the chemotaxis assay were genistein, daidzein, and coumestrol. Genistein and daidzein are strong *nod* gene inducers in *B. japonicum*, while coumestrol is a weak one (5, 15, 16). All the isoflavonoids tested were identified in soybean root extracts (26), and two of them (coumestrol and daidzein) were found in soybean root exudates (9). The hydroxycinnamic acids were of interest because of their universal presence in higher plants and their function as intermediates in the isoflavonoid biosynthesis (13). Porter et al. (27) reported the presence of a number of phenolic compounds, e.g., ferulic acid, in hydrolyzed soybean root extracts.

In addition to the chemotactic activity of the phenolic substances, their *nod* gene-inducing activity was tested to elucidate the possible correlation between chemotaxis and *nod* gene induction in *B. japonicum* which has been found for fast-growing rhizobia.

Chemotactic effect of phenolic compounds. (i) Organism and growth conditions. In the chemotaxis assay, wild-type *B. japonicum* 110*spc*4 (29) was used. The bacteria were periodically selected for high motility by three subsequent passages through swarm plates. Stock cultures of these highly motile bacteria were kept at 4°C. Bacteria used in the chemotaxis assay were grown in the defined medium of Papen and Werner (22). The following variations were made: sodium succinate (5 mM) was used as the only carbon source, and KNO_3 (5 mM) was used as the nitrogen source. The medium was buffered with KH_2PO_4 (5 mM) and Na_2HPO_4 (5 mM). The pH was adjusted to 6.8. $FeSO_4$ was added without EDTA complexation. The bacteria were incubated in 300-ml flasks with 30 ml of medium without shaking at 28°C. Static cultures were used because they favor motile and chemotactic cells (12).

(ii) Chemotaxis assay. Cells were harvested (3,000 × *g*, 5 min, 20°C) in log phase, when motility was at a maximum, washed once in potassium-HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (10 mM, pH 7.0), and diluted in the same buffer to 2×10^8 cells per ml (viable cell count). This cell suspension was used in the chemotaxis assay. The capillary assay was performed in chemotaxis chambers (21) at 29°C with an incubation time of 1 h. After

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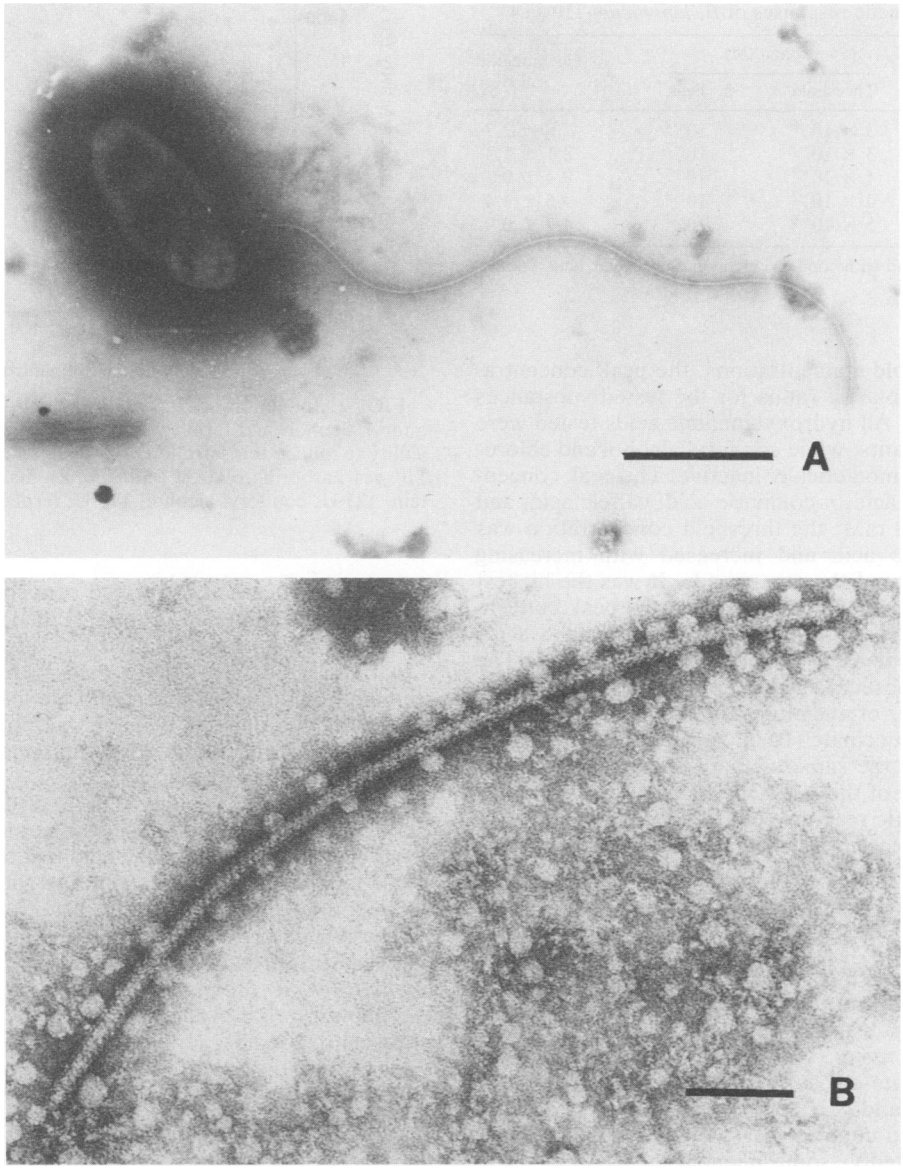

FIG. 1. (A) Electron micrograph of *B. japonicum* 110*spc*4 with subpolarly inserted complex flagellum. Negatively stained with 4% phosphorous wolfram acid, pH 7. Bar, 1 μm. (B) Isolated complex flagellum of *B. japonicum* 110*spc*4. The cross-hatched pattern of a complex flagellum is clearly discernable. Fixed with 3% glutaraldehyde. Negatively stained with 2% phosphorous wolfram acid. Bar, 100 nm.

exterior rinsing of the capillary, the cells that had entered the capillary were diluted and plated in duplicate.

All compounds tested were dissolved in HEPES buffer (10 mM, pH 7.0). The hydroxycinnamic acids were neutralized with KOH. The following compounds were tested: caffeic acid and chlorogenic acid (from 100 mM to 1 μM), cinnamic acid, *p*-coumaric acid, and ferulic acid (from 50 mM to 1 μM), sinapic acid (from 10 mM to 1 μM, coniferyl alcohol (from 1 mM to 1 μM), daidzein and genistein (from 50 μM to 1 nM), and coumestrol (from 20 μM to 1 nM). Each concentration was tested in at least two independent experiments and, within each experiment, at least in triplicate. The standard deviation for the number of cells per capillary filled with an attractant at its peak concentration was approximately 15% within one experiment and about 25% for the repetitions of the buffer control. The variation in the number of bacteria per capillary with the same attractant in different experiments was considerable. The peak concentration was affected not at all and the threshold concentration was only slightly affected by this variation. The chemotactic ratio (number of bacteria that entered the capillary tube with test substance divided by the number of bacteria that entered the capillary tube with buffer) varied somewhat from experiment to experiment. All chemotaxis data are mean values of at least two independent experiments. Chemotactic ratio, threshold concentration, and peak concentration were used according to the definitions of Mesibow and Adler (19). The threshold concentration was calculated by using linear regression to extrapolate to the background base line a double-log plot of the concentration-response curve (19).

(iii) Chemotaxis towards hydroxycinnamic acids and fla-

TABLE 1. Chemotactic responses of *B. japonicum* 110*spc*4[a]

Compound	Concn (M)		Chemotactic ratio
	Threshold	Peak	
Cinnamic acid	3×10^{-9}	10^{-2}	7.9 ± 2.7
p-Coumaric acid	3×10^{-7}	10^{-2}	8.1 ± 1.3
Caffeic acid	4×10^{-7}	10^{-2}	9.4 ± 0.3
Ferulic acid	4×10^{-7}	10^{-2}	9.5 ± 4.4
Sinapic acid	5×10^{-6}	10^{-2}	4.7 ± 0.9

[a] No response was found with coniferyl alcohol, chlorogenic acid, coumestrol, daidzein, or genistein.

vonoids. The threshold concentrations, the peak concentrations, and the chemotactic ratios for the tested substances are given in Table 1. All hydroxycinnamic acids tested were strong chemoattractants, while coniferyl alcohol and chlorogenic acids were chemotactically inactive. The peak concentrations of cinnamic acid, *p*-coumaric acid, caffeic acid, and sinapic acid were 10 mM; the threshold concentration was lowest for cinnamic acid and increased with increasing molecular complexity of the compounds. It was the highest for sinapic acid. The chemotactic ratio at the peak concentration was about the same for all hydroxycinnamic acids with the exception of a lower ratio for sinapic acid. The chemotactic ratios observed for the hydroxycinnamic acids are in the same order of magnitude as these found for yeast extract (0.1%) and succinate (10 mM). The peak concentration for succinate is the same as that for hydroxycinnamic acids, 10 mM. None of the *nod* gene-inducing isoflavonoids induced a chemotactic response of *B. japonicum* 110*spc*4.

Induction of *nod* genes by phenolic acids and flavonoids. *B. japonicum* 110*spc*4 harboring plasmid pRJ458 (14), a pRK290 derivative containing a *nodC-lacZ* fusion, was used to monitor *nod* gene induction by the compounds tested. β-Galactosidase activity was determined by the method of Miller (20), except that chlorophenol red-β-D-galactopyranoside was used as a substrate. Cells were grown for 16 h in the presence of the test substances in the same medium as used for the chemotaxis assay. Cells were permeabilized with sodium dodecyl sulfate-chloroform. As reported by Kosslak et al. (16), daidzein and genistein strongly induced β-galactosidase activity, and coumestrol was only a weak inducer (Table 2). Unexpectedly, some phenolic acids were weak

TABLE 2. β-Galactosidase expressed by *B. japonicum* 110*spc*4 harboring pRJ458 (*nodC-lacZ*) in response to hydroxycinnamic acids and derivatives and flavonoid compounds

Compound[a]	β-Galactosidase activity (U)
Cinnamic acid[b]	97
p-Coumaric acid[b]	213
Caffeic acid[b]	226
Ferulic acid[b]	362
Sinapic acid[b]	107
Coniferyl alcohol[b]	465
Chlorogenic acid[b]	429
Quinic acid[b]	106
Coumestrol[c]	416
Daidzein[c]	1,032
Genistein[c]	1,448

[a] All compounds were used at 10 μM.
[b] Background activity, 113 U.
[c] Background activity, 125 U.

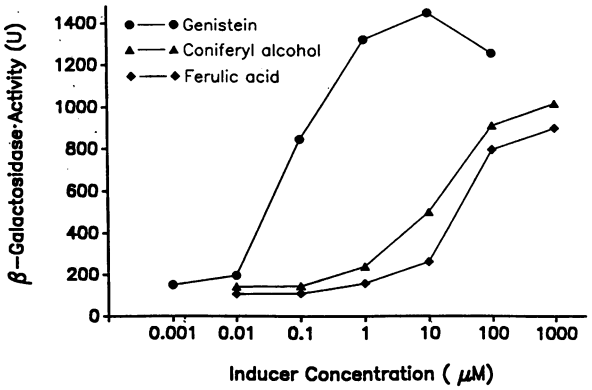

FIG. 2. Induction of *B. japonicum nod* genes by genistein, coniferyl alcohol, and ferulic acid. Compounds were tested for their ability to induce transcription of β-galactosidase from *B. japonicum* 110*spc*4 harboring pRJ458 (*nodC-lacZ*). Background levels: genistein, 125 U; coniferyl alcohol, 110 U; ferulic acid, 82 U.

nod gene inducers at the tested concentration of 10 μM. In particular, ferulic acid, coniferyl alcohol, and chlorogenic acid induced *nod* gene activity as strongly as coumestrol. Coumaric acid and caffeic acid were less active, while cinnamic acid and sinapic acid were inactive. The *nod* gene induction by coniferyl alcohol was not affected by further purification of the commercially obtained substance by thin-layer chromatography.

The dose-response curve (Fig. 2) gives further information about the concentration dependence of *nod* gene induction. Coniferyl alcohol and ferulic acid had to be applied at about 1,000-fold higher concentrations to yield the half-maximal induction level produced by genistein.

The results presented here demonstrate clear differences between fast-growing rhizobia and *B. japonicum*. While *R. meliloti* (8), *R. leguminosarum* biovar phaseoli (2), and *R. leguminosarum* biovar viciae (3) responded chemotactically towards flavonoids, no chemotactic response of *B. japonicum* towards isoflavonoids was observed. For fast-growing rhizobia, a more or less close correlation of *nod* gene induction and chemotactic effect of flavonoids has been found (2, 8). There is no indication for such a correlation in *B. japonicum*. Strong *nod* gene inducers did not elicit any chemotactic response, while weak *nod* gene inducers, such as coniferyl alcohol and ferulic acid, were either chemotactically inactive or strongly attracted *B. japonicum*.

For *R. meliloti* (6), a dual chemotaxis pathway has been found. One branch of the dual pathway seems to be specialized for the detection of nutrients, and the other branch is specialized for the detection of signals. The existence of such a dual pathway in *B. japonicum* is doubtful. The chemotactic effect of hydroxycinnamic acids probably arises not from their weak *nod* gene-inducing activity, but from their function as nutrients for *B. japonicum* (23). It is possible that the signal-specific chemotaxis pathway is missing in *B. japonicum*.

The fact that simple phenolic compounds such as hydroxycinnamic acids and coniferyl alcohol induce *nod* gene activity in *B. japonicum* deserves special attention. Among numerous other simple phenolic compounds, *p*-coumaric acid was identified as an inducer of *nodD1* from *Rhizobium* sp. strain NGR234 (18) at the same high concentration level reported here for *B. japonicum*. The *nod* gene induction of *Rhizobium* sp. strain NGR234 by simple phenolic com-

pounds was interpreted in connection with the broad host range of this strain. It might represent a stage of incomplete adaptation to the presence of the less common components of root exudates of specific legumes. By analogy, the results presented here could be interpreted with regard to the extended host range of *B. japonicum*.

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REFERENCES

1. **Adler, J.** 1988. Chemotaxis: old and new. Bot. Acta **101**:93–100.
2. **Aguilar, J. M. M., A. M. Ashby, A. J. M. Richards, G. J. Loake, M. D. Watson, and C. H. Shaw.** 1988. Chemotaxis of *Rhizobium leguminosarum* biovar *phaseoli* towards flavonoid inducers of the symbiotic nodulation genes. J. Gen. Microbiol. **134**:2741–2746.
3. **Armitage, J. P., A. Gallagher, and A. W. B. Johnston.** 1988. Comparison of the chemotactic behavior of *Rhizobium leguminosarum* with and without the nodulation plasmid. Mol. Microbiol. **2**:743–748.
4. **Ashby, A. M., M. D. Watson, G. J. Loake, and C. H. Shaw.** 1988. Ti plasmid-specified chemotaxis of *Agrobacterium tumefaciens* C58C toward *vir*-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. J. Bacteriol. **170**:4181–4187.
5. **Banfalvi, Z., A. Nieuwkoop, M. Schell, L. Besl, and G. Stacey.** 1988. Regulation of *nod* gene expression in *Bradyrhizobium japonicum*. Mol. Gen. Genet. **214**:420–424.
6. **Bergman, K., M. Gulash-Hoffee, R. E. Hovestadt, R. C. Larosiliere, P. G. Ronco II, and L. Su.** 1988. Physiology of behavioral mutants of *Rhizobium meliloti*: evidence for a dual chemotaxis pathway. J. Bacteriol. **170**:3249–3254.
7. **Boyd, A., and M. Simon.** 1982. Bacterial chemotaxis. Annu. Rev. Physiol. **44**:501–517.
8. **Caetano-Anollés, G., D. K. Crist-Estes, and W. D. Bauer.** 1988. Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. J. Bacteriol. **170**:3164–3169.
9. **D'Arcy-Lameta, A.** 1986. Study of soybean and lentil root exudates. Plant Soil **92**:113–123.
10. **Deshmane, N., and G. Stacey.** 1989. Identification of *Bradyrhizobium nod* genes involved in host-specific nodulation. J. Bacteriol. **171**:3324–3330.
11. **Firmin, J. L., K. E. Wilson, L. Rossen, and A. W. B. Johnston.** 1986. Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. Nature (London) **324**:90–92.
12. **Gaworzewska, E. T., and M. J. Carlile.** 1982. Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. J. Gen. Microbiol. **128**:1179–1188.
13. **Goodwin, T. W., and E. I. Mercer.** 1983. Introduction to plant biochemistry, 2nd ed. Pergamon Press, Oxford.
14. **Göttfert, M., S. Hitz, and H. Hennecke.** 1990. Identification of *nodS* and *nodU*, two inducible genes inserted between the *Bradyrhizobium japonicum nodYABC* and *nodIJ* genes. Mol. Plant-Microbe Interactions. **3**:308–316.
15. **Göttfert, M., J. Weber, and H. Hennecke.** 1988. Induction of a *nodA-lacZ* fusion in *Bradyrhizobium japonicum* by an isoflavone. J. Plant Physiol. **132**:394–397.
16. **Kosslak, R. M., R. Bookland, J. Barkei, H. Paaren, and E. R. Appelbaum.** 1987. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. Proc. Natl. Acad. Sci. USA **84**:7428–7432.
17. **Krupski, G., R. Götz, K. Ober, E. Pleier, and R. Schmitt.** 1985. Structure of complex flagellar filaments in *Rhizobium meliloti*. J. Bacteriol. **162**:361–366.
18. **LeStrange, K., M. Batley, J. Redmond, G. Bender, W. Lewis, B. Rolfe, and M. Nayudu.** 1988. Plant signals for nodulation by *Rhizobium*: broad and narrow host-range strategies, p. 107–108. *In* R. Palacios and D. P. S. Verma (ed.), Molecular genetics of plant-microbe interactions. APS Press, St. Paul, Minn.
19. **Mesibov, R., and J. Adler.** 1972. Chemotaxis toward amino acids in *Escherichia coli*. J. Bacteriol. **112**:315–326.
20. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Palleroni, N. J.** 1976. Chamber for bacterial chemotaxis experiments. Appl. Environ. Microbiol. **32**:729–730.
22. **Papen, H., and D. Werner.** 1980. Biphasic nitrogenase activity in *Azospirillum brasilense* in long lasting batch cultures. Arch. Microbiol. **128**:209–214.
23. **Parke, D., and L. N. Ornston.** 1984. Nutritional diversity of *Rhizobiaceae* revealed by auxanography. J. Gen. Microbiol. **130**:1743–1750.
24. **Parke, D., M. Rivelli, and L. N. Ornston.** 1985. Chemotaxis to aromatic and hydroaromatic acids: comparison of *Bradyrhizobium japonicum* and *Rhizobium trifolii*. J. Bacteriol. **163**:417–422.
25. **Peters, N. K., J. W. Frost, and S. R. Long.** 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science **233**:977–980.
26. **Porter, P. M., W. L. Banwart, and J. J. Hassett.** 1985. HPLC isolation and GC-MS identification of genistein, daidzein, and coumestrol from unhydrolyzed soybean root extracts. Environ. Exp. Bot. **25**:229–232.
27. **Porter, P. M., W. L. Banwart, and J. J. Hassett.** 1986. Phenolic acids and flavonoids in soybean root and leaf extracts. Environ. Exp. Bot. **26**:65–73.
28. **Redmond, J. W., M. Batley, M. A. Djordjevic, R. W. Innes, P. L. Kuempel, and B. G. Rolfe.** 1986. Flavones induce expression of nodulation genes in *Rhizobium*. Nature (London) **323**: 632–635.
29. **Regensburger, B., and H. Hennecke.** 1983. RNA polymerase from *Rhizobium japonicum*. Arch. Microbiol. **135**:103–109.
30. **Tsien, H.-C.** 1982. Utrastructure of free-living cell, p. 182–198. *In* W. J. Broughton (ed.), Nitrogen fixation, vol. 2: *Rhizobium*. Clarendon Press, Oxford.