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A Biovar-Specific Signal of *Rhizobium leguminosarum* bv. viciae Induces Increased Nodulation Gene-Inducing Activity in Root Exudate of *Vicia sativa* subsp. *nigra*

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Flavonoids in root exudate of leguminous plants activate the transcription of *Rhizobium* genes involved in the formation of root nodules (*nod* genes). We report that inoculation with the homologous symbiont *R. leguminosarum* bv. viciae results in an increased *nod* gene-inducing activity (Ini) in root exudate of *V. sativa* subsp. *nigra*, whereas inoculation with heterologous *Rhizobium* strains results in exudates with *nod* gene-inducing activity comparable to that of uninfected plants. Ini can be demonstrated by using either of the isogenic indicator strains containing an inducible *nod* promoter fused to the *Escherichia coli lacZ* reporter gene and the regulatory *nodD* gene of *R. leguminosarum* bv. viciae, *R. leguminosarum* bv. trifolii, or *R. meliloti*. The presence of genes *nodDABCEL* of *R. leguminosarum* bv. viciae appeared to be essential for induction of Ini. Mutation of the genes *nodI* and *nodJ* causes a delay of Ini, whereas gene *nodF* appears to be required for both the timely appearance and the maximum level of Ini activity. The *nodE* gene is responsible for the biovar specificity of induction of Ini by *Rhizobium* spp. Ini is caused by a soluble heat-stable factor of rhizobial origin. This *Rhizobium*-produced Ini factor has an apparent molecular weight between 1,000 and 10,000 and does not originate from flavonoid precursors.

Induction by *Rhizobium* bacteria of symbiotic nitrogen-fixing root nodules on leguminous plants is a host-specific process; e.g., *R. leguminosarum* bv. viciae nodulates common vetch, pea, sweet pea, and lentil but not clover or bean, whereas *R. leguminosarum* bv. trifolii nodulates only clover. Many genes required for root nodule formation (*nod* genes) by *Rhizobium* species, including those of *R. leguminosarum*, are located on a symbiosis (Sym) plasmid. In *R. leguminosarum* three types of *nod* genes have been distinguished: (i) a regulatory gene, *nodD*; (ii) the common *nod* genes, *nodABCIJ*; and (iii) the genes *nodFELMNTO*, of which the *nodE* gene is a host-specific gene which determines whether *R. leguminosarum* is able to nodulate *Vicia* or *Trifolium* plants (3–5, 14, 19, 27, 33). The NodD protein, which is required for activation of the other, inducible *nod* genes, is only active together with signal molecules, identified as flavonoids, which are exuded by the host plant roots (9, 22, 25, 46). The NodD protein shows a certain flavonoid specificity, which restricts *nod* gene induction to plants that secrete flavonoids able to activate with the NodD protein (36). Besides the constitutive *nodD* promoter, four inducible *nod* promoters have been found in *R. leguminosarum* bv. viciae, namely p*nodABCIJ*, p*nodFEL*, p*nodMN* (10, 28, 31–33), and p*nodO* (3, 5).

nod gene-inducing flavonoids have usually been identified (9, 22, 25, 46) by using bacterial strains containing a suitable *nodD* gene and an inducible *nod* promoter fused to the *Escherichia coli lacZ* reporter gene. With these constructs, *nod* gene expression can be monitored as β-galactosidase activity (17, 22, 25, 46). Up to now, the study of natural *nod* gene inducers has been restricted to the analysis of sterile seed exudates and sterile root exudates or root extracts of plants that had not previously been grown in the presence of *Rhizobium* spp. (22, 25, 46). However, in nature root exudate is not sterile, and we therefore extended our studies to exudate of plants that had been inoculated with *Rhizobium* spp. (coculture exudate). In this paper, we report that inoculation of *Vicia sativa* subsp. *nigra* plants with *R. leguminosarum* bv. viciae results in significantly increased *nod* gene-inducing activity (Ini) in coculture exudate. We show that this effect is induced by a biovar-specific extracellular signal of *R. leguminosarum* bv. viciae. The production of this signal, which is not a flavonoid, requires induction of specific *nod* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Rhizobium* strains and plasmids used in this study are listed in Table 1. Plasmids pMP254 and pMP424 are derived from pMP92 and contain the complete *nodFE* and *nodFEL* genes, respectively, of *Rhizobium leguminosarum* bv. viciae (33). Plasmids pMP258 and pMP263 contain the *nodE* gene of *R. leguminosarum* bv. viciae Sym plasmid pRL1JI and the *nodE* gene of *R. leguminosarum* bv. trifolii strain ANU843, respectively, both cloned behind the promoter of *nodABCIJ* from pRL1JI (34). Cells were grown on YMB medium (26), supplemented with 10 μg of chloramphenicol per ml (IncQ plasmids) or 2 μg of tetracycline per ml (IncP plasmids) for maintenance of the recombinant plasmids.

Plant cultures. The methods used for surface disinfection and subsequent germination of *Vicia sativa* L. subsp. *nigra* (L.) seeds have been described previously (41). Root exudates were obtained from plant cultures which were prepared as follows. Six germinated seeds with roots 1.5 cm long were transferred to a support of stainless steel wire netting located 0.5 cm above 25 ml of liquid, deposit-free Jensen medium (41) in sterile culture tubes (28 by 280 mm) plugged with cotton. The cultures were incubated for the indicated period of time at 20°C and 70% relative humidity.

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TABLE 1. *Rhizobium* strains, mutants, and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains of various cross-inoculation groups		
R. leguminosarum bv. viciae		
RBL1	Wild type	40
RBL4	Wild type	40
LPR1105	Rif^r	11
248	Wild type	16
R. leguminosarum bv. trifolii		
LPR5020	Sm^r	13
ANU843	Wild type	26
0403	Wild type	23
162S33	Wild type	Nitragin[b]
RBL51	Wild type	This article
R. leguminosarum bv. phaseoli		
1233	Wild type	15
RBL93	Wild type	This article
127K85	Wild type	Nitragin
127K17	Wild type	Nitragin
RCC3622	Wild type	Rothamsted[c]
R. meliloti		
LPR2	Wild type	12
1021	Sm^r	20
102F28	Wild type	S. R. Long
102F51	Wild type	S. R. Long
GMI2011	Wild type	37
Mutant strains		
LPR5045	*R. leguminosarum* bv. trifolii RCR5 cured of its Sym plasmid, Rif^r	12
RBL5516	RCR5 Sm^r Sp^r	24
RBL5515	RCR5 Sm^r Rif^r	24
RBL5505	RCR5 Sm^r Rif^r Sp^r	24
RBL5280	LPR5045 pMP154 pMP280	36
RBL5283	LPR5045 pMP154 pMP283	36
RBL5284	LPR5045 pMP154 pMP284	36
RBL5580	LPR5045 pRL1JI::Tn*1831*	43
RBL5601	RBL5505 pRL1JI*mep2*::Tn*5*	42
RBL5602	RBL5505 pRL1JI*nodE1*::Tn*5*	42
RBL5610	RBL5505 pRL1JI*nodD2*::Tn*5*	42
RBL5633	RBL5505 pRL1JI*nodA10*::Tn*5*	42
RBL5634	RBL5505 pRL1JI*nodB11*::Tn*5*	42
RBL5646	RBL5516 pRL1JI*nodC13*::Tn*5*	42
RBL5657	RBL5505 pRL1JI*nodF18*::Tn*5*	42
RBL5729	LPR5045 pRL1JI*nodI82*::Tn*5*	42
RBL5734	LPR5045 pRL1JI*nodJ29*::Tn*5*	42
RBL5793	LPR5045 pRL1JI*nodL589*::TnphoA	2
Plasmids		
pRL1JI	Sym plasmid of *R. leguminosarum* bv. *viciae* strain	42
pMP92	IncP cloning vector, Tc^r	36
pMP190	IncQ expression vector, Sm^r Cm^r	36
pMP154	Promoter *nodABCIJ-lacZ* fusion in pMP190[d]	36
pMP254[e]	*nodFE* genes of *R. leguminosarum* bv. viciae in pMP92	This work
pMP258[e]	*nodE* gene of *R. leguminosarum* bv. viciae behind the *nodABCIJ* promoter[c] in pMP92	34
pMP263[e]	*nodE* gene of *R. leguminosarum* bv. trifolii cloned behind promoter *nodABCIJ*[d] in pMP92	34
pMP280	*nodD* gene of *R. leguminosarum* bv. viciae inserted in pMP92	36
pMP283	*nodD* gene of *R. leguminosarum* bv. trifolii inserted in pMP92	36
pMP284	*nodD1* gene of *R. meliloti* inserted in pMP92	36
pMP424[e]	*nodFEL* genes of *R. leguminosarum* bv. viciae cloned in pMP92	This work
pMP425	*nodL* gene of *R. leguminosarum* bv. viciae cloned in pMP92	2
pMP604	FITA (Flavonoid-independent transcription activation) *nodD* gene cloned in pMP92	35

[a] Abbreviations: Rif^r, rifampicin resistance; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance.
[b] Nitragin Co., Milwaukee, Wis.
[c] Rothamsted Culture Collection, Harpenden, United Kingdom.
[d] Derived from *R. leguminosarum* bv. viciae.
[e] Details of the construction of the plasmid are given in Materials and Methods.

The light intensity at the table surface was approximately 20,000 lx (Philips TLF 60W/33 fluorescent tubes), and the day length was 16 h. The roots were not shielded from light, and no forced aeration was applied (41). Prior to cocultivation, bacteria were grown at 28°C on solid YMB medium (26) for 3 days. The cells were suspended from the plate in deposit-free Jensen medium to an A_{660} value of 0.1 and diluted 1,000-fold into the plant culture medium. The influence of extracellular bacterial factors on the *nod* gene-inducing activity of root exudate was tested after growing plants on pasteurized supernatant fluids of bacterial cultures diluted 10-fold in deposit-free Jensen medium. Root exudates were tested for the presence of bacterial contaminants after plating of 50 μl of exudate on solid TY medium (40).

Bioassay for *nod* gene-inducing activity. The presence of *nod* gene inducers was investigated by using the isogenic indicator strains RBL5280, RBL5283, and RBL5284 (Table 1), which only differ in the origin of their *nodD* genes (46). The root exudates of duplicate *V. sativa* subsp. *nigra* cultures were tested for the presence of *nod* gene-inducing activity by adding 100 μl of exudate to 2.9 ml of indicator bacteria, which were grown overnight in test tubes (17 by 180 cm) on a rotary shaker at 180 rpm at 28°C. Unless otherwise indicated, the β-galactosidase values in the tables are expressed as Miller units (21) induced by 30-fold-diluted root exudate and corrected for the background level of the indicator strain, which was 300 to 400 U. Culture supernatant fluids of *Rhizobium* cells to be tested for the presence of symbiotic signals were taken from cells grown in B medium (39), which, if appropriate, was supplemented with the *nod* gene inducer naringenin (1 μM). The fluids were tested after centrifugation for 10 min at 6,000 × *g* and pasteurization for 10 min at 80°C.

Properties of the Ini factor. Supernatant fluids of cultures of strain RBL5561 pMP604 in B medium (39) were used as a source of the Ini factor. Heat stability was tested after incubation for 10 min at 120°C. The molecular weight was estimated by ultrafiltration through YM10, YM5, and YM2 (molecular weight cutoffs, 10,000, 5,000, and 1,000, respectively) filters from Amicon Corp. (Danvers, Mass.). After filtration of 10 ml of supernatant fluid, 2 volumes of 10-ml Jensen medium were passed through the filter. The material remaining on the filter was taken up in 10 ml of Jensen medium. The filtrates and the material remaining on the filter were tested for the presence of Ini factor.

RESULTS

Influence of *R. leguminosarum* bv. viciae on *nod* gene-inducing activity in *V. sativa* subsp. *nigra* root exudate. Cocultivation with *R. leguminosarum* bv. viciae strain RBL5601 induced high levels of *nod* gene-inducing activity in *V. sativa* subsp. *nigra* root exudate, as measured by using the three indicator strains RBL5280, RBL5283, and RBL5284 (Fig. 1A). A significant effect was measured within 2 days of coculture. *nod* gene-inducing activity was maximal at day 4 and declined thereafter. We designated this phenotype as Ini (for increased *nod* gene-inducing activity). The presence of a Sym plasmid is required for Ini, since strain RBL5045, which is strain RBL5601 without Sym plasmid pRL1JI, did not increase the level of *nod* gene-inducing activity of exudate (Fig. 1B), a result similar to that observed with uninfected control plants (Fig. 1C).

Specific assay for Ini in *V. sativa* subsp. *nigra* root exudate. In contrast to exudates of *V. sativa* subsp. *nigra* plants cocultured with strain RBL5601 (Fig. 1A), exudates of uninfected *V. sativa* subsp. *nigra* plants (Fig. 1C) or plants cocultured with the Sym plasmid-cured strain LPR5045 (Fig. 1B) show little if any *nod* gene-inducing activity when strain RBL5284, harboring the *nodD1* gene of *R. meliloti*, is used as an indicator. The other two indicator strains show significant background activity (Fig. 1B and 1C). Therefore, β-galactosidase production by strain RBL5284 can be used as a specific assay for Ini (specific for the newly formed inducers), and for analysis of the genetic requirements of *Rhizobium* spp. for Ini induction. Since the *nod* genes of strain RBL5284 are not induced by the flavonoid naringenin in concentrations up to 30 μM, this strain could also be used as an indicator strain for Ini in experiments in which the ability of sterilized supernatant fluids of *Rhizobium* cells,

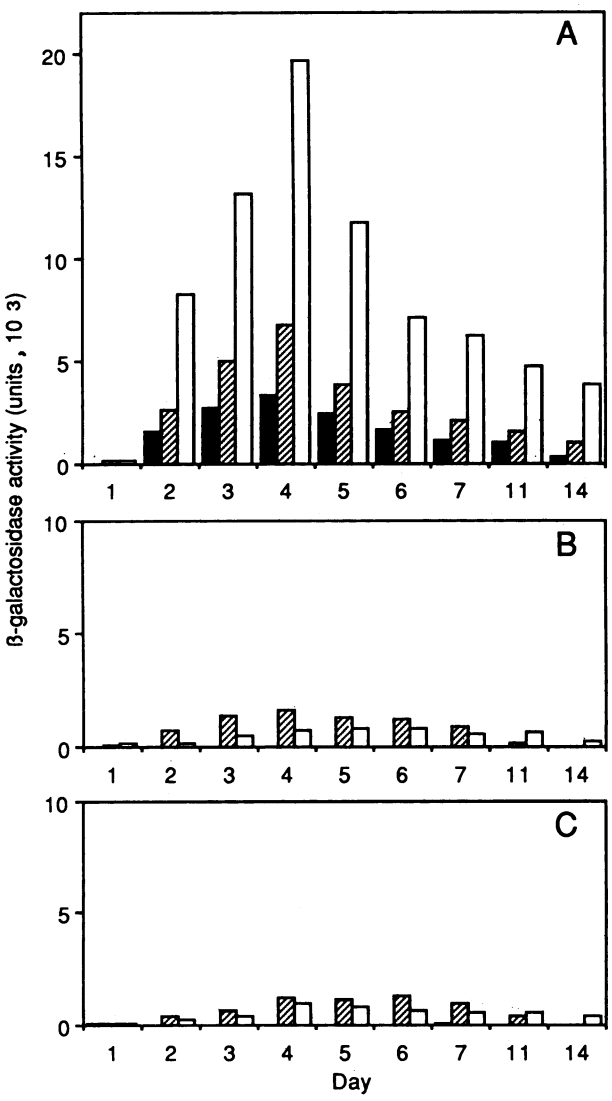

FIG. 1. Time course of *nod* gene-inducing activity of exudates of *Vicia sativa* subsp. *nigra* cultured with *Rhizobium* strain RBL5601 (A) or RBL5045 (B) or without bacteria (C). The indicator bacteria were strain RBL5284 (with *nodD1* of *R. meliloti*) (■), strain RBL5280 (with *nodD* of *R. leguminosarum* bv. viciae) (▨) and strain RBL5283 (with *nodD* of *R. leguminosarum* bv. trifolii) (□). The β-galactosidase activity was determined as described in Materials and Methods. The variation in the activities of duplicate exudates was less than 20%.

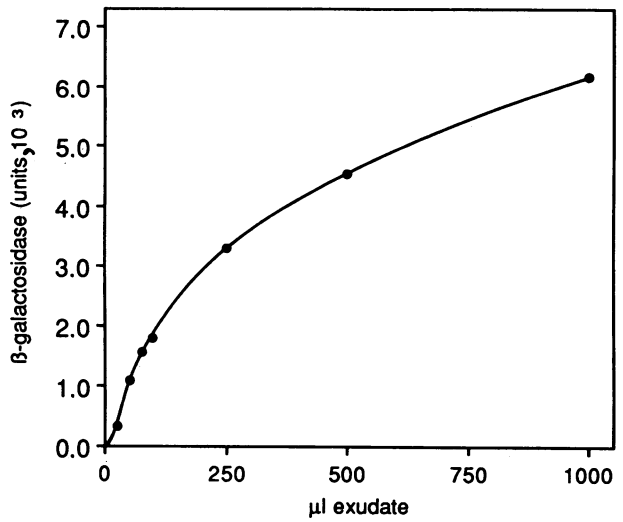

FIG. 2. Relationship between activity of Ini in exudate and resulting β-galactosidase activity. Exudate of 4-day-old *Vicia sativa* subsp. *nigra* plants, co-cultured with *R. leguminosarum* bv. viciae strain RBL5601, was produced and pasteurized as described in Materials and Methods. Various amounts were added to cultures of indicator strain RBL5284, and the resulting β-galactosidase activity was measured. Values in the figure represent averages from duplicates. The variation between duplicates remained within the points of the graph.

grown in the presence of naringenin to induce Ini, was tested. Figure 2 shows how the level of *nod* gene induction, as quantified by measuring β-galactosidase activity, increased with increasing amounts of Ini-positive root exudate present in the culture of strain RBL5284.

Ini and host specificity. Exudates of *V. sativa* subsp. *nigra* cocultured with *Rhizobium* strains from four cross-inoculation groups were investigated for Ini. All *R. leguminosarum* bv. viciae strains induced Ini after 4 and 7 days of coculture (Table 2). In contrast, strains of *R. leguminosarum* bv. trifolii, *R. leguminosarum* bv. phaseoli, and *R. meliloti* did not induce Ini after 4 days of coculture and two strains of *R. leguminosarum* bv. trifolii, strain LPR5020 and strain 0403, induced an Ini phenotype only after 7 days of coculture.

Identification of *nod* genes of *R. leguminosarum* bv. viciae required for Ini (Table 3). Involvement of *nod* genes in the induction of Ini was studied by measuring Ini in exudates of *V. sativa* subsp. *nigra* cocultured with transposon-induced *nod* mutants of *R. leguminosarum* bv. viciae. Strains with Tn*5* mutations in the common *nod* genes *nodA*, *nodB*, *nodC*, and *nodD* did not induce Ini. Strains with Tn*5* mutations in the common *nod* genes *nodI* and *nodJ* showed a delayed response: a weak Ini phenotype after 4 days of coculture, followed after 7 days of coculture by an Ini level that is normally observed with the parent strain after 4 days. Mutants with transposon mutations in the *nodF*, *nodE*, or *nodL* gene hardly induced or did not induce Ini after 4 days of coculture. However, a moderate Ini phenotype was found after 7 days.

In order to further study the requirement of *nodFEL* genes for induction of Ini, strain RBL5580, harboring a deleted pRL1JI Sym plasmid which contains the *nodFDABCIJ* genes but lacks the *nodELMNTO* genes (1) was tested. No induction of Ini was found (Table 3). The additional presence of both *nodE* and *nodL* genes (RBL5580 pMP424) resulted in almost full restoration of the Ini-inducing properties (Table 3). Strain RBL5580 pMP258, containing the *nodE* gene but lacking the *nodL* gene, did not induce Ini, showing the importance of *nodL*. In contrast, strain RBL5580 pMP425, lacking the *nodE* gene but containing a *nodL* gene, induced a diminished and delayed but significant Ini phenotype. To investigate the roles of the *nodF* and *nodE* genes in the induction of Ini, *nodF*::Tn*5* (strain RBL5657) and *nodE*::Tn*5* (strain RBL5602) mutants were complemented with an IncP plasmid harboring either a cloned *nodFE* or a cloned *nodE* gene under control of the *nodABCIJ* promoter and were tested for induction of Ini. Only in the presence of both *nodF* and *nodE* genes could Ini-inducing properties of the *nodF*::Tn*5* mutant be partially restored (Table 3). A similar partial restoration of induction of Ini occurred after induction of the cloned *nodE* gene in the *nodE*::Tn*5* mutant. Taken together, these results show that the genes *nodF*, *nodE*, and *nodL* are of crucial importance for induction of Ini by *R. leguminosarum* bv. viciae strains.

The *nodE* gene is a host-specific gene which determines whether *R. leguminosarum* is able to nodulate *Vicia* or clover plants (34). This gene therefore may be responsible for the biovar-specific restriction of induction of Ini to *R. leguminosarum* bv. viciae strains (Table 3). In order to test this hypothesis, the *nodE*::Tn*5* mutant (strain RBL5602) was complemented with an IncP plasmid harboring a cloned *nodE* gene of *R. leguminosarum* bv. trifolii under control of the *nodABCIJ* promoter. In contrast to induction of the *R. leguminosarum* bv. viciae *nodE* gene, no restoration of the Ini-inducing properties occurred by introduction of the *R. leguminosarum* bv. trifolii *nodE* gene (Table 3), demonstrating the role of the *nodE* gene in the biovar-specific induction of Ini.

Ini phenotype-inducing properties of supernatant fluids of rhizobial cultures. In order to investigate whether a soluble

TABLE 2. Influence of cocultivation with *Rhizobium* strains of various cross-inoculation groups on Ini

Strain	Ini (10^3 units of β-galactosidase)	
	4 days[a]	7 days[a]
R. leguminosarum bv. viciae		
RBL1	2.4	1.7
RBL4	2.5	1.6
LPR1105	1.4	1.6
248	2.4	1.6
RBL5601	2.2	1.1
R. leguminosarum bv. trifolii		
LPR5020	0.1	1.8
ANU843	0.1	0.0
0403	0.1	0.6
162S33	0.0	0.1
RBL51	0.0	0.1
R. leguminosarum bv. phaseoli		
1233	0.1	0.1
RBL93	0.0	0.1
127K85	0.0	0.1
127K17	0.0	0.1
RCC3622	0.0	0.2
R. meliloti		
LPR2	0.0	0.0
1021	0.0	0.1
102F28	0.0	0.1
102F51	0.0	0.2
GMI2011	0.0	0.1

[a] Ini was measured after 4 and 7 days of coculture, as described in Materials and Methods for strain RBL5284.

TABLE 3. Genetic requirements of *Rhizobium* for induction of Ini in exudate of *V. sativa* subsp. *nigra* plants

Strain	Relevant characteristics	Ini (10^3 units of β-galactosidase)	
		4 days[a]	7 days[a]
RBL5601	pRL1JI	2.4	1.7
RBL5610	pRL1JI*nodD2*::Tn*5*	0.1	0.0
RBL5633	pRL1JI*nodA10*::Tn*5*	0.1	0.0
RBL5634	pRL1JI*nodB11*::Tn*5*	0.0	0.1
RBL5646	pRL1JI*nodC13*::Tn*5*	0.0	0.0
RBL5729	pRL1JI*nodI82*::Tn*5*	1.1	2.3
RBL5734	pRL1JI*nodJ29*::Tn*5*	0.5	2.8
RBL5657	pRL1JI*nodF18*::Tn*5*	0.1	1.4
RBL5602	pRL1JI*nodE1*::Tn*5*	0.0	0.5
RBL5793	pRL1JI*nodL589*::TnphoA	0.0	0.5
RBL5580	pRL1JI::Tn1831 (Δ*nodELMNTO*)	0.0	0.1
RBL5580(pMP258)	Same as RBL5580 + *nodFE*	0.0	0.2
RBL5580(pMP424)	Same as RBL5580 + *nodFEL*	2.3	1.3
RBL5580(pMP425)	Same as RBL5580 + *nodL*[b]	0.3	1.2
RBL5657	RBL5505 pRL1JI *nodF18*::Tn*5*	0.1	1.4
RBL5657(pMP258)	Same as RBL5657 + cloned *nodE* (viciae)	0.1	1.4
RBL5657(pMP254)	Same as RBL5657 + cloned *nodFE* (viciae)	1.7	2.8
RBL5602	RBL5505 pRL1JI*nodE1*::Tn*5*	0.0	0.5
RBL5602(pMP258)	Same as RBL5602 + cloned *nodE* (viciae)	1.7	1.3
RBL5602(pMP263)	Same as RBL5602 + cloned *nodE* (trifolii)[c]	0.0	0.0

[a] Ini was measured after 4 and 7 days of coculture, as described in Materials and Methods.
[b] Plasmid pMP425 contains a promoterless *nodL* gene which is expressed at a significant level (2).
[c] In contrast to the strains RBL5602 and RBL5602(pMP258), this strain is able to form root nodules on *Trifolium repens* plants (34).

factor(s) is required for the induction of Ini, pasteurized culture supernatant fluids of *R. leguminosarum* bv. viciae strains RBL5601 and RBL5045, grown on B medium with or without the *nod* gene inducer naringenin, were tested for induction of the Ini phenotype. In contrast to results with other preparations, only culture supernatant fluids of strain RBL5601 grown in the presence of naringenin induced the Ini phenotype (Table 4). This result demonstrates that *R. leguminosarum* bv. viciae, after *nod* gene induction, produces a soluble factor(s) (designated the Ini factor), which induces increased *nod* gene-inducing activity of *V. sativa* subsp. *nigra* exudate.

The bioassay for the Ini phenotype was used for estimating the activity of the Ini factor as described in Materials and Methods. The results (Fig. 3) show that this bioassay can indeed be used as a semiquantitative test for the Ini factor.

To investigate a role of the *nod* gene inducer naringenin as a possible precursor of the Ini factor, supernatant fluids of cultures of strain RBL5561 pMP604, containing a flavonoid-independent transcription-activating *nodD* gene, grown in the absence of naringenin were tested for induction of the Ini phenotype. The results show that activation of the inducible *nod* promoters as such, and not the presence of inducer, is required for production of the Ini factor (Table 4). This result demonstrates that *nod* gene-inducing flavonoids are not precursors of the Ini factor.

TABLE 4. Presence of Ini factor in culture supernatant fluids of *R. leguminosarum* bv. viciae[a]

Source of supernatant	Induction with naringenin	10^3 Units of β-galactosidase[b]
RBL5601	−	0.0
RBL5601	+	2.7
RBL5045	−	0.0
RBL5045	+	0.0
RBL5561(pMP604)	−	4.8

[a] Supernatant fluids of *Rhizobium* cultures were obtained by centrifugation of cells (10 min at 6,000 × *g*) after growth on B medium for 24 h with (+) or without (−) 1 μM of naringenin to an A_{660} value of 0.25.
[b] Ini was determined by measuring β-galactosidase activity of strain RBL5284 in diluted exudates (1:30) of six plants grown for 4 days on a dilution (1:10) of the pasteurized *Rhizobium* culture supernatant in Jensen medium. For further details, see Materials and Methods.

Properties of the Ini factor. No significant decrease in activity of the Ini factor was found after heating of an active supernatant fluid for 10 min at 120°C. No activity of the same supernatant fluid passed through a YM2 membrane (molecular weight cutoff, 1,000), whereas half of the activity passed through a YM5 membrane (molecular weight cutoff, 5,000) and the other half stayed on top. Essentially all activity passed through a YM10 membrane (molecular weight cutoff, 10,000). This indicates either that the molecular weight of the Ini factor is close to 5,000, that more than one type of molecule has Ini factor activity, or that aggregation of the active molecule can occur.

DISCUSSION

The Ini phenotype is dependent on *nod* genes. As judged from the β-galactosidase production by *Rhizobium* indicator bacteria carrying *nodAp-lacZ* in combination with any of the

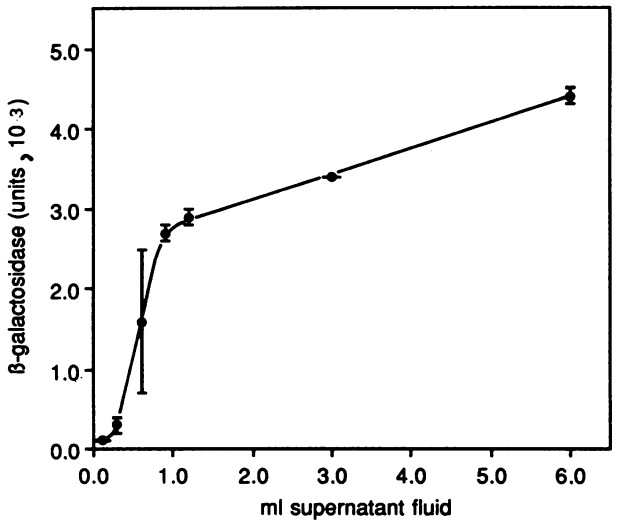

FIG. 3. Ini induction by various amounts of pasteurized culture supernatant fluids of *R. leguminosarum* bv. viciae strain RBL5601. The bacteria were grown overnight in B medium supplemented with 1 μM naringenin, and pasteurized supernatant fluids of this culture were added to duplicate cultures of *Vicia sativa* subsp. *nigra* plants. Ini (β-galactosidase activity of indicator strain RBL5284) of 4-day-old exudates was determined as described in the Materials and Methods section. Variations between the data from duplicate experiments are indicated in the graph by vertical bars.

three different *nodD* genes of various *Rhizobium* cross-inoculation groups, cocultivation of *Vicia sativa* subsp. *nigra* plants with *Rhizobium* bacteria results in the Ini phenotype, i.e., increased activity of the *nod* gene inducer in the plant exudate. The increased activity, brought about by an Ini factor secreted by the bacterium, is significant within 2 days and reaches a maximum at day 4. The activity decreases upon longer cocultivation (Fig. 1A). The Ini phenotype is dependent upon the presence of the Sym plasmid (Fig. 1B).

Analysis of various *nod* mutants and strains containing cloned *nod* DNA fragments indicated that the genes *nod ABCDEL* are absolutely required for the Ini phenotype, that mutation of the genes *nodI* and *nodJ* causes a 3-day delay, and that *nodF* is required for both a timely appearance and reaching the maximum level of Ini (Table 3).

Ini is a biovar-specific phenotype. By using an indicator strain that can specifically detect *nod* gene-inducing activity that appears in exudate upon cocultivation of the *V. sativa* subsp. *nigra* plant with *Rhizobium* bacteria (Fig. 1 and Results), it was shown that Ini is a biovar-specific phenotype (Table 2), involving the formation of inducers which are chemically different from the ones already present in sterile exudate. All five tested *R. leguminosarum* bv. viciae strains caused the phenotype after 4 days. Of the other species and biovars tested, no strains caused activity after 4 days and only two strains of bv. trifolii caused activity after 7 days of cocultivation (Table 2). The latter two strains may be more related to bv. viciae strains than the other tested strains of bv. trifolii, as in contrast to strain ANU843, strain LPR5020 forms a few "delayed" root nodules on pea plants (A. A. N. van Brussel, unpublished data). The difference between the biovars viciae and trifolii is to a major extent caused by different *nodE* genes (Table 3), a gene that recently was shown to be responsible for the difference between the two biovars in host specificity (34). It should be noted that the inability of strains of other biotypes to induce the Ini phenotype does not necessarily need to be at the level of *nodE* (35).

The degree of Ini, relative to the amount of *nod* gene-inducing activity in exudates of uninfected plants, is much greater when measured with indicator strains containing the *nodD* gene of *R. meliloti* or *R. leguminosarum* bv. trifolii than with the *nodD* gene of *R. leguminosarum* bv. viciae (Fig. 1). The biological meaning of this is not clear. One of the possibilities is that Ini is a part of a stress reaction like the thick short root (Tsr) phenotype (44). This possibility is being currently investigated.

Sequence of events resulting in the Ini phenotype (Fig. 4). The initiation of the Ini phenotype can be dissected in a number of steps. (i) Flavonoid is secreted into plant exudate, independent of the presence of *Rhizobium* bacteria. (ii) Ini factor is produced. Activation of the bacterial *nodD* gene product by flavonoids is known to be usually required for the activation of transcription of the inducible *nod* genes (33). Activation of NodD protein is also required for the production of the Ini factor (Table 3). The observation that an activated form of NodD protein rather than the presence of flavonoids is required (Table 4) indicates that a flavonoid is not a precursor of the Ini factor. Activation of the relevant *nod* genes (Table 3) results in the synthesis or secretion (Table 4) of one or more heat-stable, low-molecular-weight factors, designated as Ini factors. (iii) Synthesis or secretion of increased *nod* gene-inducing activity can be brought about by incubation of axenic *V. sativa* subsp. *nigra* plants with cell-free Ini factor. Recent analysis of the increased activity

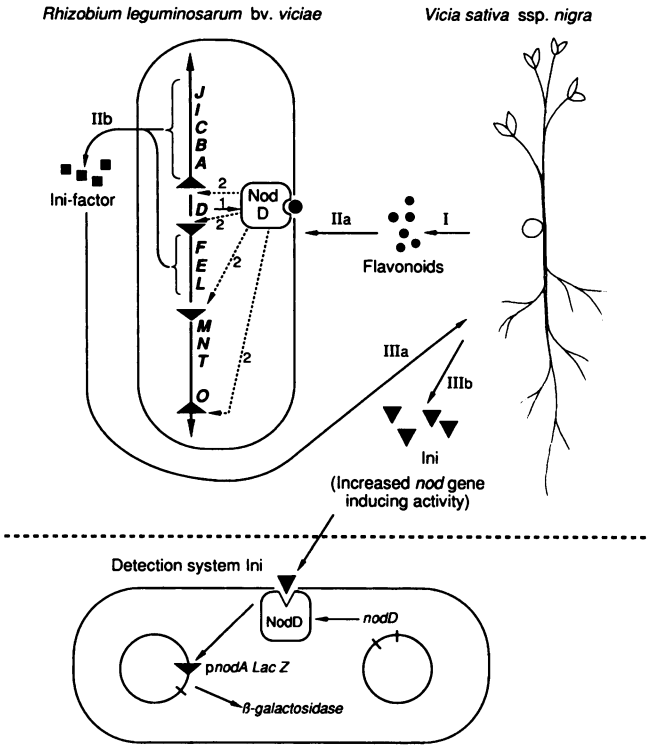

FIG. 4. Model of the sequence of events leading to the production of the Ini factor in the *R. leguminosarum* bv. viciae-*Vicia sativa* subsp. *nigra* symbiosis. For details, see text. The detection system is drawn in the lower part of the figure. Italicized letters in the bacteria indicate *nod* genes of *R. leguminosarum* bv. viciae; *lacZ* originates from *Escherichia coli*. The inducible *nod* promoters are indicated by flat triangles which point in the direction of transcription.

in the exudate has indicated that this activity is also due to flavonoid molecules (K. Recourt, unpublished data). These molecules are presently being characterized as part of a study which is meant to elucidate the molecular mechanism of the plant that is responsible for the increased activity in exudates.

Ini phenotype and nodulation. *Rhizobium* spp. require the same *nod* genes for the production of Ini factor (Table 3) as for nodulation, and the same *nod* mutants that cause a diminished and delayed Ini phenotype cause delayed nodulation (2, 6, 42). We have previously reported on the production of Tsr factor (38, 40, 41), another rhizobial low-molecular-weight, heat-stable factor. Ini factor differs from Tsr factor since the appearance of the former activity requires more *nod* genes than the genes *nodDABC* which are required for the appearance of Tsr factor activity (8, 38, 45). The genes *nodE* and *nodL* that are additionally required for the production of Ini factor are supposed to be involved in the initiation and stabilization of infection thread formation (2, 34). Therefore, it is tempting to speculate that Ini factor is involved in infection thread formation.

Scheres et al. (29) reported the induction of nodulin ENOD12 expression in pea roots by *R. leguminosarum* bv. viciae. This nodulin is involved in infection thread formation and could also be induced by cell-free supernatants of *R. leguminosarum* bv. viciae cultures. The induction of ENOD12 expression required the presence in *Rhizobium* spp. of the *nodEFDABCIJ* genes and induction of these

genes. Therefore a biovar-specific, *nodE* gene-related factor exists which causes ENOD12 expression in pea roots. Thus, *R. leguminosarum* bv. viciae with different subsets of *nod* genes produces at least three symbiotic factors, namely the Tsr factor (*nodDABC* related), the "ENOD12 factor" (*nod EFDABCIJ* related), and the Ini factor (*nodLEFDABCIJ* related). It remains to be established whether these factors are precursors of one factor, with several functions in root nodule formation, the production of which requires the whole set of *nod* genes of *R. leguminosarum* bv. viciae, or whether more than one factor is required for root nodule formation.

In the alfalfa-*R. meliloti* symbiosis, host plant-specific extracellular signals have also been found in sterilized supernatant fluids of *R. meliloti* cultures with induced *nod* genes (1, 7). The production of these signals, which induce root hair deformation (Had) on alfalfa, require the presence in *Rhizobium* spp. of the common *nod* genes and the host range genes *nodQ* and *nodH*. In the absence of the latter genes, a nonspecific *nodDABC*-related extracellular factor is formed which induces Tsr and Had on *V. sativa* subsp. *nigra* and Had on white clover, whereas Had is not induced on alfalfa by this factor. Similar factors of *R. meliloti* induce mitosis in a cell suspension culture of soybean (30). Recently (18), the *R. meliloti* host-range signal NodRm1 was identified as a sulfated β-1,4-tetrasaccharide of D-glucosamine in which three amino groups were acetylated and one was acylated with a C_{16} bisunsaturated fatty acid. NodRm1 induces specific root hair deformation on alfalfa plants.

Using the sensitive and simple assay described in this paper we are currently purifying Ini factor, which is probably related to the NodRm1 signal. Since so many *nod* genes are involved in its synthesis and secretion, the elucidation of its structure will shed light on possible biochemical functions of this factor and of the products of the *nod* genes involved.

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LITERATURE CITED

1. **Banfalvi, Z., and A. Kondorosi.** 1989. Production of root hair deformation factors by *Rhizobium meliloti* nodulation genes in *Escherichia coli*: *HsnD* (*NodH*) is involved in the plant host-specific modification of the NodABC factor. Plant Mol. Biol. **13**:1–12.
2. **Canter Cremers, H. C. J., H. P. Spaink, A. H. M. Wijfjes, E. Pees, C. A. Wijffelman, R. J. H. Okker, and B. J. J. Lugtenberg.** 1989. Additional nodulation genes on the the Sym plasmid of R. *leguminosarum* biovar *viceae*. Plant Mol. Biol. **13**:163–174.
3. **de Maagd, R. A., H. P. Spaink, E. Pees, I. H. M. Mulders, A. Wijfjes, C. A. Wijffelman, R. J. H. Okker, and B. J. J. Lugtenberg.** 1989. Localization and symbiotic function of a region on the *Rhizobium leguminosarum* Sym plasmid pRL1JI responsible for a secreted flavonoid-inducible 50-kilodalton protein. J. Bacteriol. **171**:1151–1157.
4. **Djordjevic, M. A., P. R. Schofield, and B. G. Rolfe.** 1985. Tn5 mutagenesis of *R. trifolii* host-specific nodulation genes result in mutants with altered host-range ability. Mol. Gen. Genet. **200**:463–471.
5. **Economou, A., W. D. O. Hamilton, A. W. B. Johnston, and J. A. Downie.** 1990. The *Rhizobium* nodulation gene *nodO* encodes a Ca^{++}-binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. EMBO J. **9**:349–354.
6. **Evans, I. J., and J. A. Downie.** 1986. The *nodI* gene product of *Rhizobium leguminosarum* is closely related to ATP-binding bacterial transport proteins; nucleotide sequence analysis of the *nodI* and *nodJ* genes. Gene **43**:95–101.
7. **Faucher, C., S. Camut, J. Dénarié, and G. Truchet.** 1989. The *nodH* and *nodQ* host range genes of *Rhizobium meliloti* behave as avirulence gens in *R. leguminosarum* bv. *viciae* and determine changes in the production of plant-specific extracellular signals. Mol. Plant-Microbe Interact. **2**:291–300.
8. **Faucher, C., F. Maillet, J. Vasse, C. Rosenberg, A. A. N. van Brussel, G. Truchet, and J. Dénarié.** 1988. *Rhizobium meliloti* host range *nodH* determines production of an alfalfa-specific extracellular signal. J. Bacteriol. **170**:5489–5499.
9. **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.
10. **Fisher, R. F., J. A. Swanson, J. T. Mulligan, and S. R. Long.** 1987. Extended region of nodulation genes in *Rhizobium meliloti* 1021. II. Nucleotide sequence, transcription start sites and protein products. Genetics **117**:191–201.
11. **Hooykaas, P. J. J., P. M. Klapwijk, M. P. Nuti, R. A. Schilperoort, and R. Rörsch.** 1977. Transfer of the *Agrobacterium* Ti plasmid to avirulent agrobacteria and to rhizobia ex planta. J. Gen. Microbiol. **98**:477–484.
12. **Hooykaas, P. J. J., F. G. M. Schnijdewindt, and R. A. Schilperoort.** 1982. Identification of the Sym plasmid of *Rhizobium leguminosarum* strain 1001 and its transfer to and expression in other Rhizobia and *Agrobacterium tumefaciens*. Plasmid **8**:73–82.
13. **Hooykaas, P. J. J., A. A. N. van Brussel, H. den Dulk-Ras, G. M. S. van Slogteren, and R. A. Schilperoort.** 1981. Sym plasmid of *Rhizobium trifolii* expressed in different rhizobial species and *Agrobacterium tumefaciens*. Nature (London) **291**: 351–353.
14. **Innes, R. W., P. L. Kuempel, J. Plazinski, H. C. J. Canter Cremers, B. G. Rolfe, and M. A. Djordjevic.** 1985. Plant factors induce expression of nodulation and host-range genes in R. *trifolii*. Mol. Gen. Genet. **201**:426–432.
15. **Johnston, A. W. B., J. L. Beynon, A. V. Buchanon-Wollaston, S. M. Setchell, P. R. Hirsch, J. E. Beringer.** 1978. High frequency transfer of nodulation ability between strains and species of *Rhizobium*. Nature (London) **276**:634–636.
16. **Josey, D. P., J. L. Beynon, A. W. B. Johnston, and J. E. Beringer.** 1979. Strain identification in *Rhizobium* using intrinsic antibiotic resistance. J. Appl. Bacteriol. **46**:343–350.
17. **Kosslak, R. M., R. Bookland, J. Barkei, H. E. 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.
18. **Lerouge, P., P. Roche, C. Faucher, F. Maillet, G. Truchet, J. C. Promé, and J. Dénarié.** 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature (London) **344**:781–784.
19. **Long, S. R.** 1989. *Rhizobium*-legume nodulation: life together in the underground. Cell **56**:203–214.
20. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149**:114–122.
21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Peters, N. K., J. W. Frost, and S. R. Long.** 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* genes. Science **233**:977–980.
23. **Philip-Hollingworth, S., R. I. Hollingsworth, and F. B. Dazzo.** 1989. Host-range related structural features of the acidic extracellular polysaccharides of *Rhizobium trifolii* and *Rhizobium leguminosarum*. J. Biol. Chem. **264**:1461–1466.
24. **Priem, W. J. E., and C. A. Wijffelman.** 1984. Selection of strains

cured of the *Rhizobium leguminosarum* Sym plasmid pRL1JI by using small bacteriocin. FEMS Microbiol. Lett. **25**:247–251.

25. **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.
26. **Rolfe, B. G., P. M. Gresshoff, and J. Shine.** 1982. Rapid screening method for symbiotic mutants of *R. leguminosarum* biovar *trifolii* and white clover plants. Plant Sci. Lett. **19**:227–284.
27. **Rossen, L., C. A. Shearman, A. W. B. Johnston, and J. A. Downie.** 1985. The *nodD* gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the *nodA,B,C* genes. EMBO J. **4**:3369–3373.
28. **Rostas, K., E. Kondorosi, B. Horvath, A. Simoncsits, and A. Kondorosi.** 1986. Conservation of extended promoter regions of nodulation genes in *Rhizobium*. Proc. Natl. Acad. Sci. USA **83**:1757–1761.
29. **Scheres, B., C. Van de Wiel, A. Zalensky, B. Horvath, H. Spaink, H. Van Eck, F. Zwartkruis, A.-M. Wolters, T. Gloudemans, A. Van Kammen, and T. Bisseling.** 1990. The ENOD12 gene product is involved in the infection process during the pea-*Rhizobium* interaction. Cell **60**:281–294.
30. **Schmidt, J., R. Wingender, M. John, U. Wieneke, and J. Schell.** 1988. *Rhizobium meliloti nodA* and *nodB* genes are involved in generating compounds that stimulate mitosis of plant cells. Proc. Natl. Acad. Sci. USA **85**:8578–8582.
31. **Schofield, P. R., and J. M. Watson.** 1986. DNA sequence of *Rhizobium trifolii* nodulation genes reveals a reiterated and potentially regulatory sequence preceding *nodABC* and *nodFE*. Nucleic Acids Res. **14**:2891–2903.
32. **Shearman, C. A., L. Rossen, A. W. B. Johnston, and J. A. Downie.** 1986. The *Rhizobium leguminosarum* nodulation gene *nodF* encodes a polypeptide similar to acyl-carrier protein and is regulated by *nodD* plus a factor in pea root exudate. EMBO J. **5**:647–652.
33. **Spaink, H. P., R. J. H. Okker, C. A. Wijffelman, E. Pees, and B. J. Lugtenberg.** 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol. Biol. **9**:27–39.
34. **Spaink, H. P., J. Weinman, M. A. Djordjevic, C. A. Wijffelman, R. J. H. Okker, and B. J. J. Lugtenberg.** 1989. Genetic analysis and cellular localization of the *Rhizobium* host specificity-determining NodE protein. EMBO J. **8**:2811–2818.
35. **Spaink, H. P., C. A. Wijffelman, R. J. Okker, and B. J. J. Lugtenberg.** 1989. Localization of functional regions of the *Rhizobium nodD* product using hybrid *nodD* genes. Plant Mol. Biol. **12**:59–73.
36. **Spaink, H. P., C. A. Wijffelman, E. Pees, R. J. H. Okker, and B. J. J. Lugtenberg.** 1987. *Rhizobium* nodulation gene *nodD* as a determinant of host specificity. Nature (London) **328**:337–340.
37. **Truchet, G., F. Debellé, J. Vasse, B. Terzaghi, A.-M. Garnerone, C. Rosenberg, J. Batut, F. Maillet, and J. Dénarié.** 1985. Identification of *Rhizobium meliloti* pSym2011 region controlling the host specificity of root hair curling and nodulation. J. Bacteriol. **164**:1200–1210.
38. **Van Brussel, A. A. N., E. Pees, H. P. Spaink, T. Tak, C. A. Wijffelman, R. J. H. Okker, G. Truchet, and B. J. J. Lugtenberg.** 1988. Correlation between *Rhizobium leguminosarum nod* genes and nodulation phenotypes on *Vicia*, p. 483. *In* H. Bothe, F. J. de Bruijn, and W. E. Newton (ed.), Nitrogen fixation: hundred years after. Gustav Fisher Verlag, Stuttgart, Federal Republic of Germany.
39. **van Brussel, A. A. N., K. Planqué, and A. Quispel.** 1977. The wall of *Rhizobium* leguminosarum in bacteroid and free-living forms. J. Gen. Microbiol. **101**:51–56.
40. **van Brussel, A. A. N., T. Tak, A. Wetselaar, E. Pees, and C. A. Wijffelman.** 1982. Small leguminosae as test plants for nodulation of *Rhizobium leguminosarum* and other *Rhizobia* and *Agrobacteria* harbouring a leguminosarum plasmid. Plant Sci. Lett. **27**:317–325.
41. **van Brussel, A. A. N., S. A. J. Zaat, H. C. J. Canter Cremers, C. A. Wijffelman, E. Pees, T. Tak, and B. J. J. Lugtenberg.** 1986. Role of plant root exudate and Sym plasmid-localized nodulation genes in the synthesis by *Rhizobium leguminosarum* of Tsr factor, which causes thick and short roots on common vetch. J. Bacteriol. **165**:517–522.
42. **Wijffelman, C. A., E. Pees, A. A. N. van Brussel, R. J. H. Okker, and B. J. J. Lugtenberg.** 1985. Genetic and functional analysis of the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Arch. Microbiol. **143**:225–232.
43. **Wijffelman, C. A., B. Zaat, H. Spaink, I. Mulders, A. A. N. van Brussel, R. Okker, R. De Maagd, and B. J. J. Lugtenberg.** 1986. Induction of *Rhizobium nod* genes by flavonoids: differential adaptation of promoter, *nodD* gene and inducers for various cross-inoculation groups, p. 123–135. *In* B. Lugtenberg (ed.), Recognition in microbe-plant symbiotic and pathogenic interactions. NATO ASI series, vol. H 4. Springer Verlag, Berlin.
44. **Zaat, S. A. J., A. A. N. van Brussel, T. Tak, B. J. J. Lugtenberg, and J. W. Kijne.** 1989. The ethylene-inhibitor aminoethoxyvinylglycine restores normal nodulation by *Rhizobium leguminosarum* biovar. *viciae* on *Vicia sativa* subsp. *nigra* by suppressing the 'Thick and short roots' phenotype. Planta **177**:141–150.
45. **Zaat, S. A. J., A. A. N. van Brussel, T. Tak, E. Pees, and B. J. J. Lugtenberg.** 1987. Flavonoids induce *Rhizobium leguminosarum* to produce *nodDABC* gene-related factors that cause thick, short roots and root hair responses on common vetch. J. Bacteriol. **169**:3388–3391.
46. **Zaat, S. A. J., C. A. Wijffelman, I. H. M. Mulders, A. A. N. van Brussel, and B. J. J. Lugtenberg.** 1988. Root exudates of various host plants of *Rhizobium leguminosarum* contain different sets of inducers of *Rhizobium* nodulation genes. Plant Physiol. **86**:1298–1303.