Biological Activity of *Rhizobium* sp. NGR234 Nod-factors on *Macroptilium atropurpureum*

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Received 16 July 1993. Accepted 8 October 1993.

The broad host range of *Rhizobium* sp. NGR234 is based mainly on its ability to secrete a family of lipo-oligosaccharide Nod factors. To monitor Nod-factor purification, we used the small seeded legume *Macroptilium atropurpureum,* which responds evenly and consistently to Nod factors. At concentrations between $\cong 10^{-11}$ M and 10^{-9} M, this response takes the form of deformation of the root hairs. Higher concentrations ($\cong 10^{-9}$ to 10^{-7} M), provoked profound "shepherd's crook" type curling of the root hairs. Similar concentrations of Nod factors of *Bradyrhizobium japonicum, Rhizobium leguminosarum,* and *R. meliloti* also provoked marked curling of the root hairs, but the latter two species are unable to nodulate *Macroptilium.* On the other hand, plant hormones, hormone-like substances, inhibitors of hormone action, as well as substituents of Nod factors were without effect in this bioassay. We thus conclude that only Nod factors are capable of inducing shepherd's crook type curling of *Macroptilium* root hairs. Perturbations in the auxin-cytokinin balance induced "pseudo" nodulation on *M. atropurpureum,* as did NodNGR factors at concentrations between 10^{-7} and 10^{-6} M. Concomitant inoculation of *Macroptilium* with a NodABC$^-$ mutant of NGR234 and sulfated NodNGR factors (NodNGR[S]) gave rise to plants that slowly greened, showing that the NodNGR factors permitted entry of the Nod$^-$ mutant into the roots.

Additional keywords: auxin transport inhibitors, cytokinins, nitrogen fixation, nodules.

In 1900, Hiltner showed that aqueous, bacteria-free filtrates from mature *Pisum sativum* nodules contain a substance that induces root hair formation (Hai) and deformation of the root hairs (Had) of peas. Much later, McCoy (1932) confirmed that this Had activity is contained in bacteria-free filtrates of separately grown rhizobia. Since then, a number of observers have published similar findings (Thornton 1936; Thornton and Nicol 1936; Sahlman and Fåhraeus 1962; Haack 1964; Li and Hubbell 1969; Yao and Vincent 1969; Hubbell 1970; Solheim and Raa 1973; Yao and Vincent 1976). Although the nature of the substance was never properly elucidated, it was clear that it was not indoleacetic acid and that it possessed a marked degree of legume-*Rhizobium* specificity (Sahlman and Fåhraeus 1962; von Stenz 1962; Fåhraeus and Ljunggren 1968; Broughton 1978).

In 1982, van Brussel *et al.* revived interest in these factors by showing that the Sym plasmid of *R. leguminosarum* modified the growth of *Vicia sativa* roots when transferred to heterologous rhizobia (or to *Agrobacterium tumefaciens*). On *V. sativa* (but not *V. hirsuta*) the *Agrobacterium-Rhizobium* (pJB5JI) transconjugants provoked formation of thicker and shorter roots (therefore termed the Tsr phenotype) (van Brussel *et al.* 1982). Further studies showed that Tn*5*-insertions in the *nodB* and *nodC* genes of *R. leguminosarum* abolished the Tsr effect (van Brussel *et al.* 1986), while Bhuvaneswari and Solheim (1985), Erwin and Hubbel (1985), and van Brussel *et al.* (1986) described the partial purification of the factors. Shortly afterwards, Zaat *et al.* (1987), showed that the genetic and physiological requirements for Tsr and for the production the hair-deforming factor are the same (Tsr is simply a 10-fold less sensitive response than Had) and that both are induced by flavonoid regulators of *nodD*.

In extending these observations to the symbiosis between *Medicago sativa* and *R. meliloti,* Faucher *et al.* (1988) suggested that the *nodABC* operon determines the production of a "common" factor that is modified by the product of the *R. meliloti* host-specificity gene *nodH* into an *M. sativa*-specific factor. By expressing the *nodABC* genes in *Escherichia coli* (with or without *nodH*), Banfalvi and Kondorosi (1989) lent support to the Faucher *et al.* (1988) hypothesis. Faucher *et al.* (1989) showed that another *R. meliloti* host-specificity gene, *nodQ,* is also involved in modifying the common factor in a host-specific manner. Further studies have shown that the wild-type *R. meliloti* factors are sulfated β-1,4-oligosaccharides of *N*-acetyl-D-glucosamine acylated with a C_{16}*bis*-unsaturated fatty acid (Lerouge *et al.* 1990; Roche *et al.* 1991*a*; Schultze *et al.* 1992). Factors of *Azorhizobium caulinodans* (Mergaert *et al.* 1993), *Bradyrhizobium japonicum* (Sanjuan *et al.* 1992), *Rhizobium* sp. NGR234 (Price *et al.* 1992), and *R. leguminosarum* bv. *viciae* (Spaink *et al.* 1991) clearly belong to the same class of molecules. As these factors are products of the nodulation genes, they are termed Nod factors (Roche *et al.* 1991b).

Nod factors provoke Had at extremely low concentrations. They also possess a degree of host-specificity and are mitogenic. Nod factors of *R. leguminosarum* and *R. meliloti* induce the formation of pre-infection structures (van Brussel *et*

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MPMI Vol. 6, No. 6, 1994, pp. 764-774

al. 1992) and nodules on the roots of certain legumes (Truchet *et al.* 1991). Since nodule initiation is a property often associated with plant hormones (Allen *et al.* 1953; Hirsch *et al.* 1989), and cytokinins stimulate mitosis, we compared the properties of Nod factors with those of plant hormones. In most of the work reported here, we used the Nod factors of *Rhizobium* sp. NGR234 (Price *et al.* 1992), which is known to nodulate more than 75 genera of legumes (S. G. Pueppke and W. J. Broughton, unpublished).

As *Macroptilium atropurpureum* responds consistently and unambiguously to Nod factors, it was used as the test plant. Using a simple Had/Hac bioassay, we compared the activity of Nod factors of various (brady)rhizobia to those of the living bacteria from which they were extracted. Higher

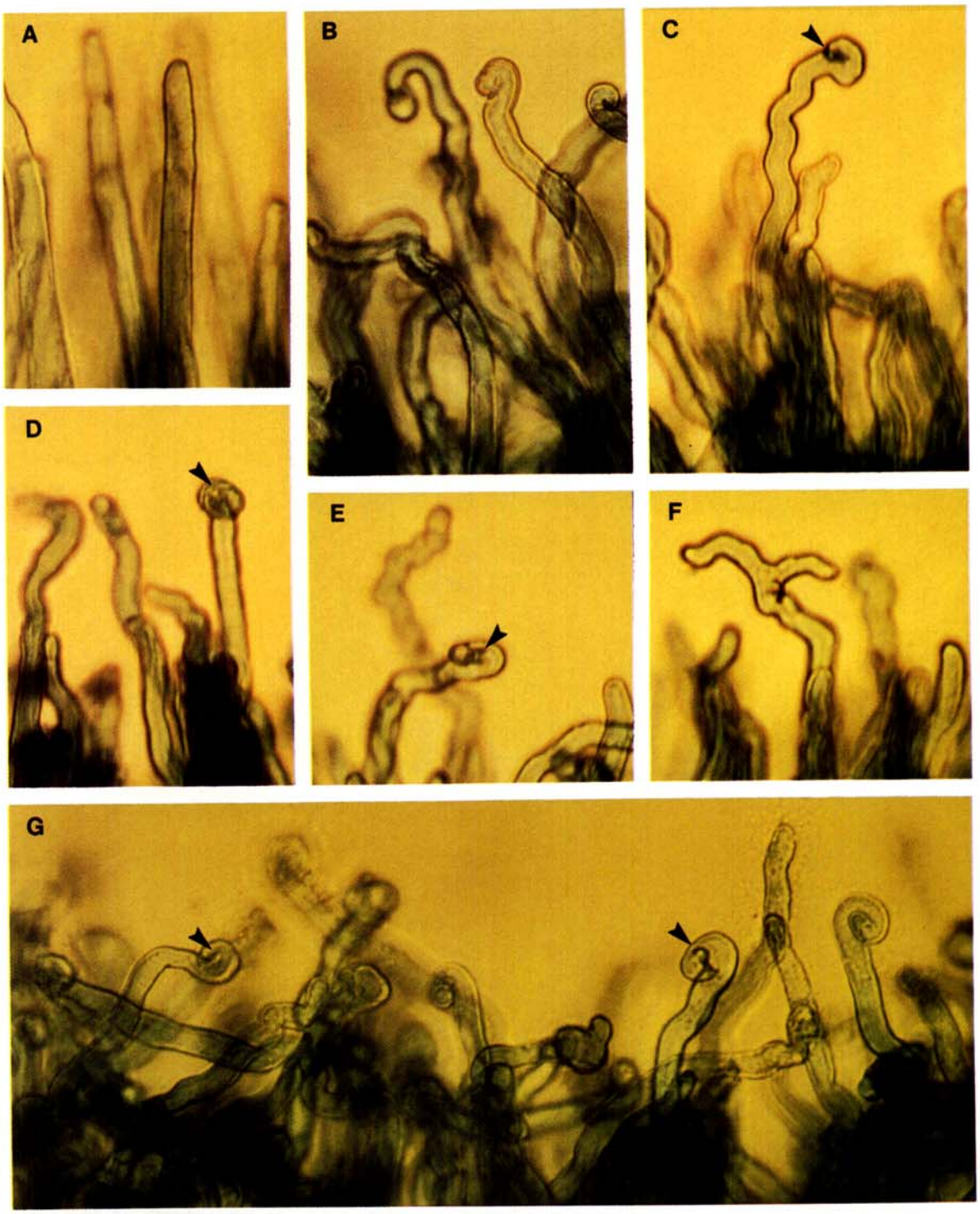
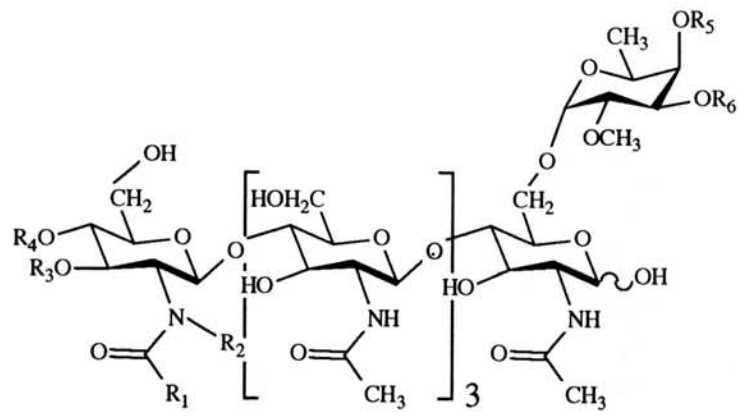


Fig. 1. Effect of partially purified NodNGR factors on deformation (Had) and curling (Hac) of *Macroptilium atropurpureum* root-hairs. **A,** control; **B–E,** various forms of Hac, showing "shepherd's crook" formation; **F,** Had (branching), and **G,** positive control with *Rhizobium* sp. NGR234. Arrows point to the hyaline "bright" spots characteristic of shepherd's crooks.

A



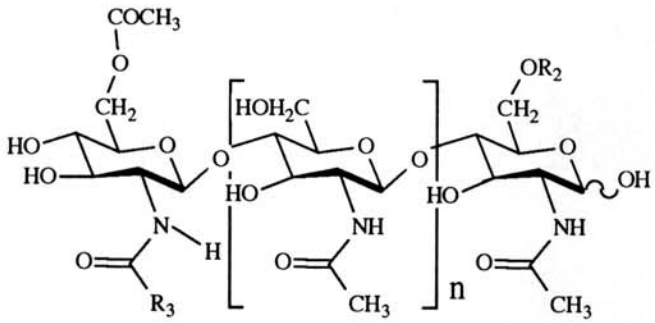
***Rhizobium* sp. NGR234**

R1 = C18:1 (Δ11) or C16:0
R2 = CH_3
R3 = carbamoyl or H
R4 = carbamoyl or H
R5 = acetate or H
R6 = sulphate or H

Bradyrhizobium japonicum

R1 = C18:1 (Δ9)
R2 = H
R3 = H
R4 = H
R5 = H
R6 = H

B



Rhizobium leguminosarum

R1 = C18:4 (Δ2,4,6,11) or C18:1 (Δ11)
R2 = H
n = 2 or 3

Rhizobium meliloti

R1 = C16:2 (Δ2,9)
R2 = sulphate
n = 2 or 3

Fig. 2. Structures of the lipo-oligosaccharide Nod factors used in this study. The nomenclature follows that proposed by Roche *et al.* (1991b) and the structures were taken from Price *et al.* (1992) (*Rhizobium* sp. NGR234), Sanjuan *et al.* (1992) (*Bradyrhizobium japonicum*), Spaink *et al.* (1991) (*R. leguminosarum*), and Lerouge *et al.* (1990) (*R. meliloti*).

Table 1. Effect of Nod factors of (brady)rhizobia, on the deformation (Had) and curling (Hac) of root hairs of *Macroptilium atropurpureum*[a]

Nod factors	Concentration[b] 10^{-7} M	10^{-9} M	10^{-11} M	Detection limit
NodNGR[Ac]	Hac[c]	Hac	$Had^{+/-}$	$\cong 5 \times 10^{-10}$ M
NodNGR[OH]	Hac	Hac	$Had^{-/+}$	$\cong 10^{-10}$ M
NodNGR[S]	Hac, inh. roots[c]	Hac, inh. roots[c]	Had	$\cong 10^{-11}$ M
NodBjV(18:1)	Hac	$Hac^{+/-}$	0	
NodRlIV(18:4, Ac)	Hac	$Hac^{+/-}$	0	
NodRmIV,V(Ac,S)	Hac	Hac	0	

[a] Concentrations of Nod factors shown are those that bathed the roots.
[b] Structures of the Nod factors are shown in Fig. 2.
[c] Inhibition of root growth.

Table 2. Derivates of NodNGR factors and related compounds that were inactive (at concentrations between 10^{-5} and 10^{-11} M) in causing deformation or curling of the root hairs of *Macroptilium atropurpureum*

Analogues of *N*-acetyl-D-glucosamine (*N*-Ac-Glu)
N-Acetyl-D-glucosamine
N-Acetyl-D-glucosamine-3-sulfate
N-Acetyl-D-glucosamine-6-sulfate
N-Acetyl-D-glutamic acid
Analogues of 2-*O*-methyl-L-fucose
α-D(+)Fucose
Octyl-β-D-glucopyranoside
2′-Fucosyllactose
Fucoidan (0.1, 0.01, and 0.001%, w/v)[a]
Oligomers of *N*-acetyl-D-glucosamine
Dimer = $[N\text{-Ac-Glu}]_2$
Trimer = $[N\text{-Ac-Glu}]_3$
Tetramer = $[N\text{-Ac-Glu}]_4$
Pentamer = $[N\text{-Ac-Glu}]_5$
Acyl chains
Palmitic acid = C_{16}:0
Palmitoleic acid = C_{16}:1
cis-Vaccenic acid = *cis*-C_{18}:1
trans-Vaccenic acid = *trans*-C_{18}:1
BF-7 (a diglycosyl diacylglyceride glycolipid)[b]

[a] Concentrations of fucoidan solutions are given in percent.
[b] Described in Orgambide *et al.* (1992).

concentrations of NodNGR factors were also used to test Nod-factor effects on the induction of nodulation.

RESULTS

Are Nod factors toxic?

Before undertaking large-scale trials with substances of unknown pharmacological properties, we used the brine shrimp (*Artemia salina* (Leach)) bioassay for toxicity (Beloz 1992). Concentrations of NodNGR[S] of $\leq 10^{-6}$ M had no effect on development or mortality of this crustacean (data not shown). We thus concluded that NodNGR factors, at the concentrations used in the following experiments, are not inherently toxic in the short term.

Had and Hac bioassay with *M. atropurpureum*.

To screen Nod-factor preparations, we sought a small-seeded, rapidly germinating legume with long, even root hairs among the hosts of *Rhizobium* sp. NGR234. *Macroptilium atropurpureum* Urb. 'Siratro' is not only simple to cultivate but responds consistently and unambiguously to Nod factors. Often the response takes the form of true shepherd's crook type curling (Fig. 1), defined as meeting the following criteria (Hiltner 1900; Fåhraeus 1957; Yao and Vincent 1969). First, the curvature of the root hair tip must be ≥360°. Second, a bright "hyaline" spot should be visible in the middle of the curve. Third, to avoid confusion with root hair tip deformations due to contact with adjacent root hairs, these deformations should be seen in isolated root hairs (Truchet *et al.* 1985). Inoculation of *M. atropurpureum* with *Rhizobium* sp. NGR234 provokes the formation of abundant shepherd's crooks (cf. Fig. 1A and G), but partially purified NodNGR factors also produce them (Fig. 1B–E). Other types of deformation, including branching (Fig. 1F), were also observed. Had was visible in the range from 10^{-11} M to 10^{-7} M, and Hac from $\cong 10^{-9}$ M to 10^{-7} M. Even higher concentrations of NodNGR factors severely stunted root growth. Unfortunately, dilution represents the only means of quantifying the response to Nod factors, but statistical analysis of the Had data allows a semi-quantitative measurement of activity (Price *et al.* 1992). Sulfated Nod factors (NodNGR[S])(Fig. 2) are more active than acetylated ones (NodNGR[Ac]), which in turn are more active than the forms that are neither acetylated nor sulfated (NodNGR[OH]) in this system (Table 1). At concentrations below $\cong 10^{-9}$ M, true Hac was no longer apparent, but other forms of deformation were visible.

Specificity of Hac in *M. atropurpureum*.

In addition to sensitivity, any screening method must be specific to the substance in question. To test this, we assayed the response of *M. atropurpureum* root hairs, first to Nod factors of other rhizobia (Table 1), then to different derivatives of Nod factors (Table 2), and finally to various plant hormones as well as inhibitors of hormone action (Table 3). Surprisingly, all Nod-factor preparations that were available until the end of 1992 (Fig. 2) caused shepherd's crook type curling of the root hairs (Fig. 3). This included Nod factors from *B. japonicum* (strain USDA110), which nodulates *M. atropurpureum,* as well as those from *R. leguminosarum* bv. *viciae* (strain RBL5560) and *R. meliloti* (strain 2011) which do not (Table 4). In accordance with previously published data (Price *et al.* 1992), it should be noted however, that 10–100 times higher concentrations of Nod factors from heterologous rhizobia are necessary to achieve the same degree of curling as that given by NodNGR factors.

On the other hand, no other substance tested was able to provoke Hac. Among the compounds that were inactive were all the plant hormones and inhibitors of hormone action (Table 3), as well as BF-7, a diglycosyl diacylglyceride gly-

colipid that is symbiotically active in *Trifolium repens* (Orgambide *et al.* 1993)(Table 2). At high concentrations (10^{-5} M), some of these compounds were toxic (e.g., acetylsalicyclic acid), whereas others restricted growth of the roots and/or root hairs (Ethepon, L-α-[2-aminoethoxyvinyl]-glycine, thiabendazole [TIBA], and Tubulozole-C). Only indoleacetic acid [IAA] stimulated the formation of root hairs. Between 10^{-9} M and 10^{-5} M, there was a linear relationship between shoot-growth and gibberellic acid concentration, suggesting that the *Macroptilium* test is also a simple and sensitive bioassay for gibberellins. In summary, the M. atropurpureum bioassay meets our two primary requirements—it is extremely sensitive and it is specific to Nod factors.

Complete NodNGR factors are required for biological activity.

If parts of Nod-factor molecules were biologically active, this would simplify a number of molecular procedures, including development of highly radioactive probes for receptor-binding studies. Accordingly, we tested the Had activity of all commercially available components of NodNGR factors using the *M. atropurpureum* bioassay (Table 2). None of the constituents of the factors were active, suggesting that only the entire molecule is able to play a symbiotic role.

Auxin transport inhibitors and cytokinins induce "pseudo" nodules on *Macroptilium.*

As mentioned in the introduction, nodule initiation is a property associated with plant hormones. Since hormones were without effect on root hair deformation but either stimulated development of root hairs (IAA) or inhibited root growth (naphthalene acetic acid, various cytokinins), we tested the ability of auxins and cytokinins as well as inhibitors of hormone action to induce pseudo nodules. At concentrations between 10^{-5} and 10^{-6} M, a number of cytokinins, as well as (naphthyl)phthalamic acid (NAB) and TIBA (at 10^{-5} M), inhibitors of auxin transport, provoked the formation of pseudo nodules on roots of *M. atropurpureum* (Fig. 4). Although the comparative uptake rates of cytokinins and auxin transport inhibitors are not known, our data suggest that cytokinins are more effective than transport inhibitors in promoting pseudo nodule formation. Simultaneous addition of the NGRΔ*nodABC*, and the auxin transport inhibitors or cytokinins did not allow the Nod$^-$ mutant to penetrate the roots of *Macroptilium.*

NodNGR factors also induce pseudo nodules on *M. atropurpureum.*

As a number of reports have shown that Nod factors induce pseudo nodule formation on the roots of various legumes, we treated the roots of *M. atropurpureum* with all three NodNGR factors (Table 1, Fig. 2). In these experiments, concentrations of NodNGR factors of $\geq 10^{-7}$ M elicited pseudo nodules. Organization of the vascular bundles within the pseudo nodules resembled those in nodules rather than in roots, and for that reason we called these structures pseudo nodules (Fig. 4A). In the pseudo nodules, the cortical cells that normally contain bacteroids are filled with amyloplasts containing starch grains (Fig. 4B).

Table 3. Effect of plant hormones, inhibitors of hormone action, and related substances on root growth, deformation of the root hairs, and pseudo nodule initiation on *Macroptilium atropurpureum*

Hormones[a]	Effect on plant growth[b]	Had[c]	Pseudo nodules[b,d]	Mode of action[b]
Abscisic acid	...	0	NT	...
Aspirin	toxic	0	NT	...
Auxins				
IAA	stim. root-hairs	0	NT	...
NAA	inh. roots	0	0	...
Cytokinins				
BAP	inh. roots	0	10^{-5} to 10^{-6}	...
2iP	inh. roots	0	10^{-5} to 10^{-6}	...
Kinetin	inh. roots	0	10^{-5} to 10^{-6}	...
Zeatin	...	0	NT	...
Zeatin riboside	...	0	NT	...
Ethylene				
Ethepon	inh. roots	0	NT	...
Gibberellins				
GA_3	stim. shoots (10^{-5} to 10^{-9})	0	NT	...
GA_7	stim. shoots (10^{-5} to 10^{-9})	0	NT	...
Methyl jasmonate	...	0	0	...
Triacontanol	...	0	NT	...
Inhibitors				
AVG	inh. root hairs	0	NT	Inh. C_2H_4
CCC	...	0	0	Inh. GA
NPA	inh. shoots	0	few	Inh. IAA transport
TIBA	inh. roots	0	few	Inh. IAA transport
Tubulozole-C	inh. roots, root hairs (10^{-5} to 10^{-7})	0	NT	Inh. tubulin

Aspirin = acetylsalicyclic acid, IAA = indole-3-acetic acid; NAA = α-napthalaneacetic acid, BAP = 6-benzylaminopurine, 2iP = 6-(γ,γ-dimethylallylamino)purine, AVG = L-α-(2-aminoethoxyvinyl)-glycine, CCC = chlorocholine chloride, NPA = *N*-1-(naphthyl)phthalamic acid, and TIBA = 2,3,5-triiodobenzoic acid.

[b] Concentrations in parentheses are the molar concentrations that gave the effect, and were 10^{-5} unless otherwise stated. Inh. = inhibition, stim. = stimulation. ... = No effect.

[c] Had = Hair-deformation activity was tested at concentrations between 10^{-11} and 10^{-5} M.

[d] NT = Not tested.

NodNGR factors allow a NodABC⁻ mutant to enter *Macroptilium* roots.

If NodNGR factors cause the extreme type of deformation (Hac) of *Macroptilium* root hairs normally associated with nodulation and provoke pseudo nodule formation, it seemed reasonable to ask if they would also allow bacteria that are inherently unable to penetrate the roots to nodulate. As the *nodABC* genes are thought to be responsible for the production of a "common" Nod factor described in the introduction, we used a mutant of *Rhizobium* sp. NGR234 which has had the *nodABC* genes deleted (NGRΔ*nodABC*, Table 5). Not only is NGRΔ*nodABC* unable to produce Nod factors or to provoke root hair deformation, it is Nod⁻ on all plants tested (including *M. atropurpureum*; Relić *et al.* 1993).

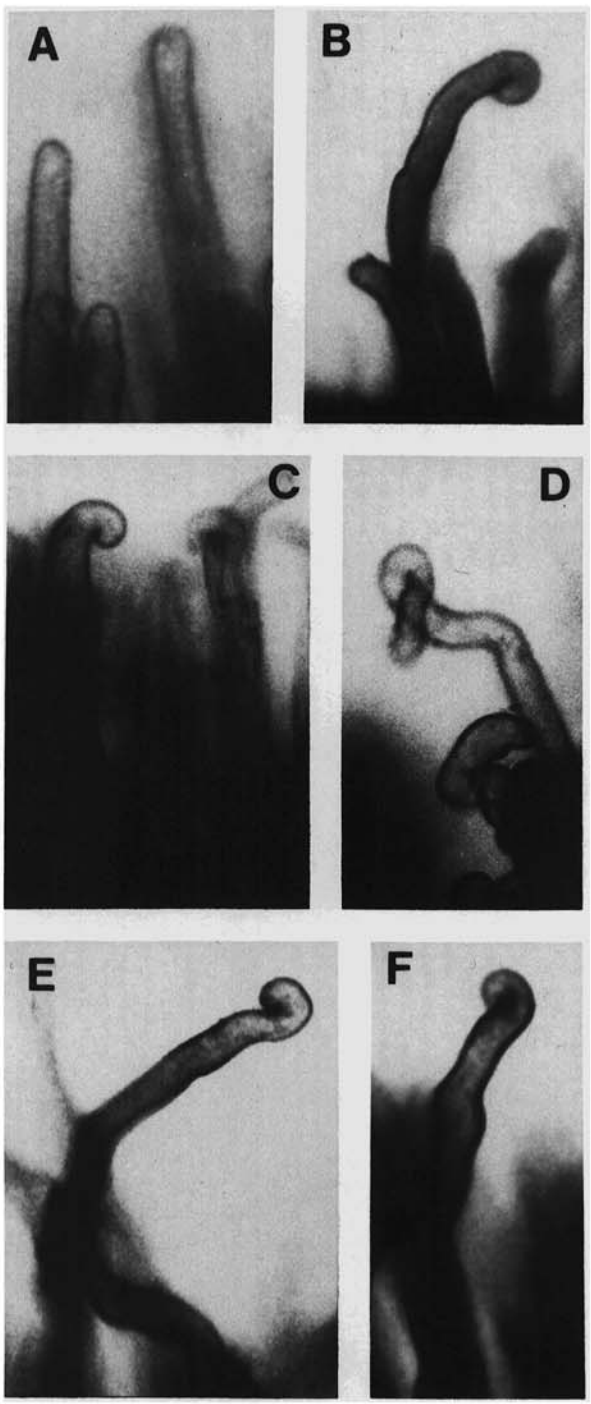


Fig. 3. Effect of 10^{-7} M Nod-factors of *Bradyrhizobium japonicum* [NodBjV(18:1)], *Rhizobium leguminosarum* [NodRlIV(18:4, Ac)], and *R. meliloti* [NodRmIV,V(Ac,S)] on deformation (Had) and curling (Hac) of root-hairs of *Macroptilium*. **A,** Control; **B,** NodRmIV/V(Ac,S); **C and D,** NodRl(18:4,Ac), and **E and F,** NodBjV(18:1).

At concentrations of $\cong 10^{-7}$ M, all NodNGR factors (Table 1, Fig. 2) induced pseudo nodules as well as Fix⁺ nodules that contained NGRΔ*nodABC* (≅8 and 4 nodules/plant, respectively). About 30 days after inoculation, most of the plants slowly turned green at the apex (Fig. 5). In turn, some of the lower leaves greened from the apex downwards, showing that nitrogen fixation had occurred. Export of nitrogen from the roots to developing tissue in the apices is a characteristic of plants recovering from starvation and shows that nodulation only occurred when the demand for nitrogen was high. As NGRΔ*nodABC* is impaired only in its ability to produce Nod factors, this suggests that once within the nodule, the intact nitrogen fixation genes function normally. Light and electron microscopic comparisons of wild-type and Nod-factor-induced Fix⁺ nodules (Fig. 4) showed that the main differences were related to the size of the nodule. Probably in an attempt to supply nitrogen to the whole plant, the few nodules induced by Nod factors were larger than those produced by NGR234. Some evidence of bacteroidal degradation was also apparent (cf. Fig. 4D and G), but this comparison may not be valid, because the exact age of the nodules is unknown. In any event, Figure 5 clearly proves that the bacteroids were capable of nitrogen fixation.

DISCUSSION

M. atropurpureum is widely used to confirm the third of Koch's postulates (Broughton *et al.* 1975; Broughton and John 1979; Somasegaran and Hoben 1985), implying that it possesses a broad host range. Although later studies have shown that *Vigna unguiculata* is even more promiscuous, *M. atropurpureum* should nevertheless be considered as a plant with a broad, but not infinite, capacity to nodulate (Lewin *et al.* 1987). At first sight, this might explain why all the Nod factors tested caused marked shepherd's crook type curling of the root hairs. Yet, only *B. japonicum* and *Rhizobium* sp. NGR234 are able to nodulate *Macroptilium.* This would seem

Table 4. Nodulation capacity of the wild-type *(Brady)rhizobium* sp. used in this study[a]

	Host				
	Glycine	***Macroptilium***	***Medicago***	***Vicia***	***Vigna***
B. japonicum USDA110	Fix⁺	Fix⁺	Nod⁻	Nod⁻	Fix⁺
Rhizobium sp. NGR234	Fix⁻	Fix⁺	Nod⁻	Nod⁻	Fix⁺
R. leguminosarum v. *viciae* RBL5560	Nod⁻	Nod⁻	Nod⁻	Fix⁺	Nod⁻
R. meliloti RCR2011	Nod⁻	Nod⁻	Fix⁺	Nod⁻	Nod⁻

[a] Ability of the strains to nodulate was tested in Magenta jars under standard conditions (Lewin *et al.* 1990). Nod⁻ means without nodules, and Fix⁺ that the nodules formed and actively fixed nitrogen.

to question the dogma that markedly curled root hairs foretell nodule initiation (e.g., Vincent 1974). Yet the Nod-factor concentrations that provoke shepherd's crook formation on heterologous and homologous legumes are considerably different. In other words, these data do not allow us to distinguish between the possibilities that shepherd's crook formation is an essential but not completely specific step in nodulation (Broughton 1978), or that specificity is largely regulated by Nod-factor levels.

Our data confirm the findings of Allen *et al.* (1953) that substituted benzoic acids induce nodule-like structures on the roots of various legumes. In extending their observations, we show here that direct application of cytokinins (BAP, 2iP, or kinetin), or inhibitors of auxin transport (NPA and TIBA) produce the same effect on *Macroptilium*. Nod factors, at 10- to 100-times lower concentrations, elicit pseudo nodules on *Macroptilium*, as well as on *Medicago sativa* (Truchet *et al.* 1991) and *Sesbania caulidulans* (Mergaert *et al.* 1993). In

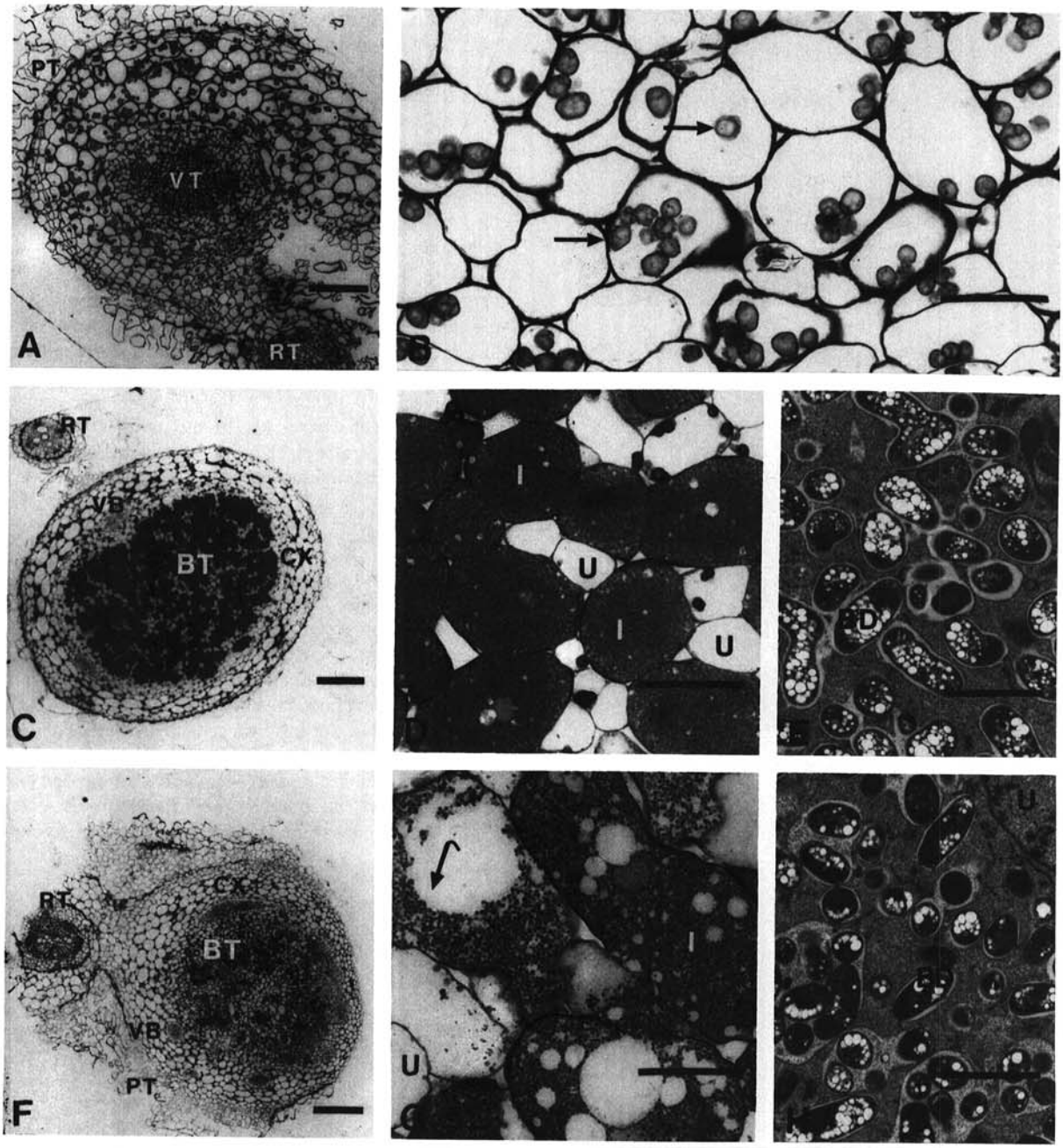


Fig. 4. Morphology of nodules produced on *Macroptilium atropurpureum* by treatment with *Rhizobium* sp. NGR234 Nod factors (A and B), by inoculation with *Rhizobium* species NGR234 (C–E), and by concomitant application of the Nod$^-$ mutant NGRΔ*nodABC* and 10^{-7} M NodNGR[S] (F–H). **A, C, and F,** Low-magnification light micrographs of whole nodules (bars = 150, 200, and 200 μm, respectively); **B, D, and G,** High-magnification micrographs of nodule sections (bars = 50 μm); and **E and H,** Electron micrographs of bacteroidal tissue (bars = 2 μm). BD = bacteroid, BT = bacteroidal tissue, CX = nodule cortex, I = infected cells, PT = protective tissue, RT = root, VB = vascular bundle, VT = vascular tissue, and U = uninfected cells. Arrows point to amyloplasts containing starch grains. Curved arrows point to degradation of bacteroids.

other words, Nod factors and increases in the cytokinin-auxin balance have the same effect on the formation of pseudo nodules. As Nod factors share properties with plant hormones, they may be regarded as "hormone-like" molecules.

Nod factors possess another essential characteristic that is not shared with plant hormones, however, the ability to curl root hairs. Most probably, curling of this type traps rhizobia within the inner folds of the curl and permits a later, extremely specific step to occur—the entry of rhizobia into the infection thread. Close proximity of the homologous rhizobia to plant membranes is probably necessary for penetration, and shepherd's crook type curling of the root hairs provides the environment in which it occurs. Nod factors are thus hormone-like substances with two symbiotically important properties—they cause extreme curling of the root hairs (and so entrap rhizobia) and produce the same effect on roots as a perturbation in the cytokinin-auxin balance. Under certain conditions, these combined properties allow *Rhizobium* sp. NGR 234 to enter the roots of *M. atropurpureum*.

MATERIALS AND METHODS

Bacteria.

(*Brady*)*rhizobium* strains, along with their relevant characteristics, are listed in Table 5. Microbiological techniques were performed as described in Lewin *et al.* (1990).

Plants.

Seeds of *Gylcine max* (L.) Merr. 'Preston' and *B. japonicum* USDA110 (Sp^R) were provided by Petra Schmidt, Botanik, der Philipps-Universität, Marburg, Germany; *M. atropurpureum* Urb. 'Siratro' were purchased from Rawlings Seeds, Orpington, Kent, UK; H. Meyer z.A. (Max-Planck-Institut für Züchtungsforschung, Cologne, Germany) provided seeds of *Medicago sativa* L. 'Cardinal'; *Vicia sativa* L. subsp. *nigra* (L.) Ehrh. was from INRA, Lusignan, France; and seeds of *Vigna unguiculata* (L.) Walp. 'Red Caloona' were from Rawlings Seeds.

Table 5. *Rhizobium* strains used in this study.

Designation	Characteristics[a]	Source or reference
Rhizobium sp.		
NGR234 (Rif^R)	Rif^R derivative of strain NGR234	Lewin *et al.* 1990
NGRΔ*nodABC*	*nodABC* deletion mutant of NGR234, Rif^R, Sp^R	Price *et al.* 1992
R. leguminosarum bv. *viciae*		
RBL5560	Wild-type LPR5045 containing pSym pJB5JI, Sm^R	Spaink *et al.* 1992
R. meliloti		
RCR2011	Wild-type, Sp^R	Rosenberg *et al.* 1981
B. japonicum		
USDA110	Wild-type, Sp^R	Hahn and Hennecke 1984

[a] pSym = symbiotic plasmid; Rif = rifampicin (50 $\mu g \cdot ml^{-1}$); Sp = spectinomycin (50 $\mu g \cdot m^{-1}$), and; Sm = streptomycin (50 $\mu g \cdot ml^{-1}$).

Large-scale Nod-factor production.

NodNGR factors were isolated from apigenin-induced (10^{-6} M) culture medium of the overproducing strain, NGR234(pA28). Solid-phase extraction onto large-scale C_{18} reverse-phase columns followed by preparative HPLC on the same support was performed essentially as described in Price *et al.* (1992).

Plant assays.

Assays for Had activity were performed as described in Price *et al.* (1992). Nodulation tests were performed in Magenta jars (Lewin *et al.* 1990). To exclude light, they were painted black, and the vermiculite was covered with black plastic beads. All plants were grown at a daytime temperature of 30° C, a night temperature of 20° C, and a light phase of 16 hr (including a 1-hr stepped "sunrise" and a 1-hr stepped "sunset"; maximum intensity of illumination was 350 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ photosynthetically active radiation). Two replicate jars, each containing four seedlings, were used per treatment. Controls included plants treated with wild-type NGR234, NGRΔ*nodABC* (both at 10^7 cfu/plant), and 1 ml B & D solution (Broughton and Dilworth 1971). Treatment consisted of the addition, 3 days after planting, of NodNGR factors directly to the B & D solution, to a final concentration of 10^{-7} M. Daily addition of H_2O maintained the level of the B & D medium in the Magenta jars at 250 ml. Plants were harvested 50 days after treatment. After sterilization (10 min in 70%, v/v, ethanol), nodules were rolled on tryptone-yeast (TY) agar to check for surface contamination and divided in two. A small portion of the inner bacteroidal tissue was removed from both halves and streaked out on TY. If colonies developed, they were restreaked on TY containing the appropriate antibiotics, used to inoculate *M. atropurpureum* held in Magenta jars, and raised in liquid culture for the isolation of total DNA. In the 14 nodules examined this way, only NGRΔ*nodABC* was found as demonstrated by 1) possession of the appropriate antibiotic resistances, 2) absence of nodulation on *Macroptilium*, and 3) the shift or elimination of bands in Southern blots, depending on whether a radioactive probe spanning *nodABC* or a probe internal to *nodABC* was used (data not shown). The rest of the nodule halves were prepared for light and electron microscopy (Golinowski *et al.* 1987). These experiments were repeated twice.

ACKNOWLEDGMENTS

WJB would like to acknowledge the late D. O. Norris's suggestion that *M. atropurpureum*, due to its broad host range and small seeds, is well suited to nodulation tests. We are extremely grateful to Slobodan Relić for his invaluable help with the large-scale production of NodNGR factors. Jean Dénarié, Herman Spaink, and Gary Stacey generously donated Nod factors of *R. meliloti*, *R. leguminosarum*, and *B. japonicum*, respectively. Frank Dazzo, René Decorzant, and Ann Hirsch graciously provided samples of BF-7 methyljasmonate and NPA. Thomas Boller and Christian Staehelin are thanked for making the tetramers and pentamers of *N*-acetyl-D-glucosamine available. Petra Schmidt and Heiner Meyer z.A. kindly provided seeds. Neil Price first showed that BAP and kinetin induce pseudo nodules on the roots of *Macroptilium*. We wish to thank Thomas Boller, Dora Gerber, Saïd Jabbouri, Danielle Promé, and Christian Staehelin for their help with many aspects of this work. Rémy Fellay and Andrea Krause corrected the manuscript. Financial assistance was provided by the Erna och Victor Hasselblads Stiftlese, the Conseil Régional Midi-Pyrénées, the Fonds National Suisse de la Recherche Scientifique (Projects 31-30950.91 and 31-36454.92), and the Université de Genève.

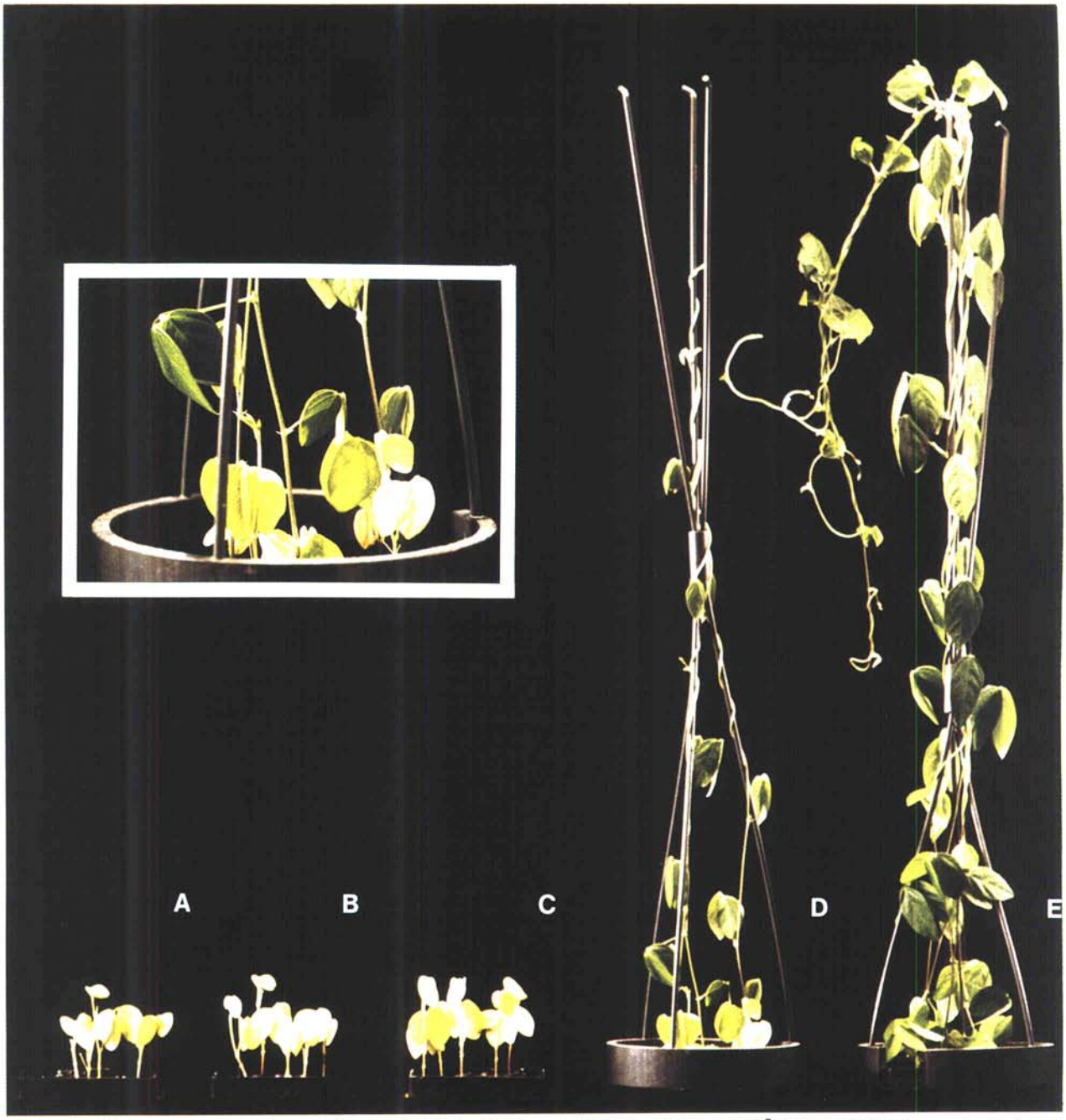


Fig. 5. *Macroptilium atropurpureum* plants co-inoculated with the Nod$^-$ mutant, NGRΔ*nodABC*, and 10^{-7} M NodNGR[S] factors. **A,** Un-inoculated, negative control; **B,** NodNGR[S] factors alone; **C,** NGRΔ*nodABC* alone; **D,** concomitant inoculation with NGRΔ*nodABC* and NodNGR[S] factors; and **E,** positive control inoculated with *Rhizobium* sp. NGR234. Insert shows the manner in which the plants greened from the top down, as well as one of the four plants that failed to nodulate. The photograph was taken 50 days after planting.

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