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Root Hair Deformation Activity of Nodulation Factors and Their Fate on *Vicia sativa*[1]

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We used a semiquantitative root hair deformation assay for *Vicia sativa* (vetch) to study the activity of *Rhizobium leguminosarum* bv *viciae* nodulation (Nod) factors. Five to 10 min of Nod factor-root interaction appears to be sufficient to induce root hair deformation. The first deformation is visible within 1 h, and after 3 h about 80% of the root hairs in a small susceptible zone of the root are deformed. This zone encompasses root hairs that have almost reached their maximal size. The Nod factor accumulates preferentially to epidermal cells of the young part of the root, but is not restricted to the susceptible zone. In the interaction with roots, the glucosamine backbone of Nod factors is shortened, presumably by chitinases. NodRlv-IV(C18:4,Ac) is more stable than NodRlv-V(C18:4,Ac). No correlation was found between Nod factor degradation and susceptibility. Degradation occurs both in the susceptible zone and in the mature zone. Moreover, degradation is not affected by NH_4NO_3 and is similar in vetch and in the nonhost alfalfa (*Medicago sativa*).

The *Rhizobium*-legume interaction starts with the exchange of signal molecules between both partners. Flavonoids secreted by the roots of the host plant trigger the expression of the nodulation (*nod*) genes of *Rhizobium*, resulting in the synthesis of specific lipooligosaccharides named Nod factors (Fisher and Long, 1992; Spaink, 1992; Dénarié and Cullimore, 1993).

The structure of the major Nod factor produced by *Rhizobium meliloti* was the first to be elucidated (Lerouge et al., 1990). At present, the structure of Nod factors produced by several *Rhizobium* species has been determined (Dénarié and Cullimore, 1993), showing that all Nod factors are molecules consisting of a β-1,4-linked *N*-acetyl-D-glucosamine backbone varying in length between three and five sugar units. The nonreducing terminal sugar moiety is substituted on the C-2 position with a fatty acid group, the structure of which is variable. Depending on the *Rhizobium* species, additional substitutions on the terminal sugar residues are present (Spaink, 1992; Dénarié and Cullimore, 1993).

The Nod factors produced by *Rhizobium leguminosarum* bv *viciae* have an *O*-acetyl group at the C-6 position on the nonreducing terminal sugar residue; there is no substitution present on the reducing sugar. The acyl moiety is a C_{18} chain with either four or one double bond(s) (Spaink et al., 1991).

Nod factor nomenclature is based on the similarities of all published Nod factors (Spaink, 1992). In this nomenclature the species indication (e.g. Rlv for *R. leguminosarum* bv *viciae*) is followed by a roman numeral, referring to the number of glucosamine units, and a term in parentheses indicating the length of the acyl chain and degree of unsaturation and substitutions on nonreducing and reducing sugar residues [e.g. NodRlv-V(C18:4,Ac)].

After the discovery of the rhizobial Nod factors, the extent to which these molecules could induce steps of the nodulation process was studied (Fisher and Long, 1992; Spaink, 1992; Dénarié and Cullimore, 1993). In vetch, root hair deformation is induced by both C18:4- and C18:1-containing NodRlv factors, whereas cortical cell divisions and preinfection thread formation can be induced only by NodRlv factors containing a C18:4 acyl group (Spaink et al., 1991; Van Brussel et al., 1992). The Nod factor-induced nodule primordia in vetch become arrested at an early stage in development (Vijn et al., 1993), but in some plant species Nod factors are able to induce the development of complete nodule structures (Fisher and Long, 1992; Dénarié and Cullimore, 1993). Purified Nod factors are not able to promote the formation of genuine infection threads, but they induce the expression of the infection-related early nodulin genes in pea as well as in transgenic *Medicago varia* roots (Horvath et al., 1993; Pichon et al., 1993).

Thus, Nod factors play a key role in the induction of early steps of nodulation, and therefore it is important to elucidate the mechanisms by which these *Rhizobium* signal molecules induce these early steps. For this purpose, it is essential to use assays in which the activity of Nod factors can be perceived, preferably shortly after application of the factor. According to this criterion the best assays available are the depolarization of alfalfa root hair membranes, which occurs within 10 min (Ehrhardt et al., 1992), and the induction of ENOD12 gene expression in the root epidermis of alfalfa, which is induced in about 3 h (Pichon et al., 1993).

Root hair deformation has frequently been used to study the activity of Nod factors because it is a simple assay. In an assay developed for clover (*Trifolium repens* L.), root hair

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Abbreviations: CHAPS, (3-[3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; Nod, nodulation.

deformation was scored 3 h after inoculation with *R. leguminosarum* bv *trifolii* and these deformed root hairs were present in a zone located 2 to 5 mm from the tip (Bhuvaneswari and Solheim, 1985). However, in all but one (Firmin et al., 1993) of the studies to determine Nod factor activity, root hair deformation was examined several days after addition of Nod factor (Lerouge et al., 1990; Spaink et al., 1991; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; Mergaert et al., 1993).

We adapted the assay described by Bhuvaneswari and Solheim (1985) to study *Vicia sativa* root hair deformation and we show that the first deformation can be observed after only 1 h and that maximal deformation is established after 3 h. A period of Nod factor-root contact as short as 10 min is sufficient to induce root hair deformation. This root hair deformation assay can be used as a semiquantitative assay by which the activity of different Nod factors is compared. Furthermore, we compared the fate of radioactive Nod factors containing four or five GlcNAc residues and studied the effect of NH_4NO_3 on deformation and on the the fate of Nod factors.

MATERIALS AND METHODS

Plant Material

Vicia sativa subspecies *nigra* seeds were sterilized and germinated as described by Van Brussel et al. (1982). Germinated seeds were transferred to modified Fåhraeus slides (Bhuvaneswari and Solheim, 1985) in small trays containing Fåhraeus medium (Fåhraeus, 1957). Each slide contained 1 mL of medium and six plants. The plants were grown at 22°C with a 16-h light period for 2 d. After 1 d the slides were transferred to Petri dishes and put in a slanted position and the medium was replaced by fresh medium. After 2 d the roots had grown about 1 cm and they were used in a deformation assay. The same procedure was followed for the alfalfa seeds.

Root Hair Deformation Assay

Before applying Nod factors the roots of all plants were examined microscopically and Fåhraeus slides containing plants with deformed root hairs were discarded. The medium was replaced by medium containing Nod factor and the Fåhraeus slide was incubated in a Petri dish at 22°C. After 3 h the roots were microscopically examined. The amount of deformed root hairs in the susceptible zone was determined and deformation was rated 0, 1, or 2 corresponding to 0–20%, 20–60%, and >60% deformed root hairs. At least two Fåhraeus slides were used for each incubation and deformation was scored blindly by two persons.

The length of the root hairs in the susceptible zone was determined over a 24-h period using an ocular micrometer to ascertain if these hairs were still growing during the time in which they were susceptible to Nod factor. Root hair deformation was followed in time using a video camera. A Fåhraeus slide was placed under the microscope and the susceptible zone was located. After exchanging the medium with medium containing Nod factor, some root hairs in the susceptible zone were followed in time by continuous recording for 4 h.

Isolation and Labeling of Nod Factors

NodRlv factors were purified using the overproducing *R. leguminosarum* bv *viciae* strain LPR5045(.pIJ1089) ("wild-type") (Spaink et al., 1991), and the concentration of purified Nod factors was determined according to Spaink et al. (1991). NodRlv-V(C18:4,Ac) (1 mg) was incubated with 1 unit of exochitinase (*Streptomyces griseus*, Sigma) for 24 h under conditions described by the manufacturer. The resulting material was applied to a reversed-phase HPLC column (5 μm, 4 × 250 mm, Pharmacia LKB) with acetonitrile:water (30:70) as a mobile phase (flow rate, 0.7 mL/min). NodRlv-III(C18:4,Ac) and NodRlv-II(C18:4,Ac) were purified by elution with an acetonitrile:water gradient (30:70 to 65:35 in 25 min and monitoring the eluate at 206 and 303 nm). The structure of the purified compounds was confirmed by MS analyses.

NodRlv factors were labeled in vivo by adding 0.5 mCi of [^{14}C]acetate (56 mCi mmol^{-1}) to a 200-mL naringinin-induced culture of the overproducing *R. leguminosarum* bv *viciae* (LPR5045.pIJ1089) strain as described by Spaink et al. (1991). By using labeled acetate it is likely that both the acyl moiety and the sugar backbone are labeled. The specific activity was about 26 mCi mmol^{-1} for each Nod factor.

The [^{3}H]NodRlv-V(C18:0,Ac) compound was made (at the Commissariat a l'Energy Atomique, Gif sur Yvette, France) by reducing the acyl group of NodRlv-V(C18:4,Ac) with Pd/C under a $^3H_2$ atmosphere by a procedure derived from the method described by Roche et al. (1991b). NodRlv-V(C18:4,Ac) was reduced in a solution of *n*-butanol:water (1:1) instead of using methanol, and the catalyst was extracted with DMSO after labeling instead of filtering the catalyst and collecting the methanol filtrate. The specific activity of this compound was about 200 Ci mmol^{-1}.

Nod Factor Extraction and TLC Analysis

Fåhraeus slides containing vetch plants were incubated with 6 × 10^{-7} M (15,000 cpm mL^{-1}) [^{14}C]NodRlv factor for 10, 60, or 180 min. At each time point the medium of three slides was collected and extracted with *n*-butanol. The roots of the plants were washed five times with water, the roots were collected and ground in liquid nitrogen in a small mortar, and the ground material was extracted with *n*-butanol. The *n*-butanol fractions were evaporated and redissolved in *n*-butanol. The complete root extract and one-third of the medium extract were analyzed by TLC. The same procedure was used for Fåhraeus slides containing alfalfa plants or Fåhraeus slides incubated with [^{3}H]NodRlv-V(C18:0,Ac).

To determine the degradation in the susceptible and the mature zone of the vetch roots, 100 plants were harvested 3 h after application of 5 × 10^{-9} M (10^6 cpm mL^{-1}) [^{3}H]NodRlv-V(C18:0,Ac), and segments of 2 to 3 mm of the susceptible zone and the zone containing mature root hairs were isolated using a dissecting microscope and *n*-butanol extracted as described above. The extracts were analyzed by TLC.

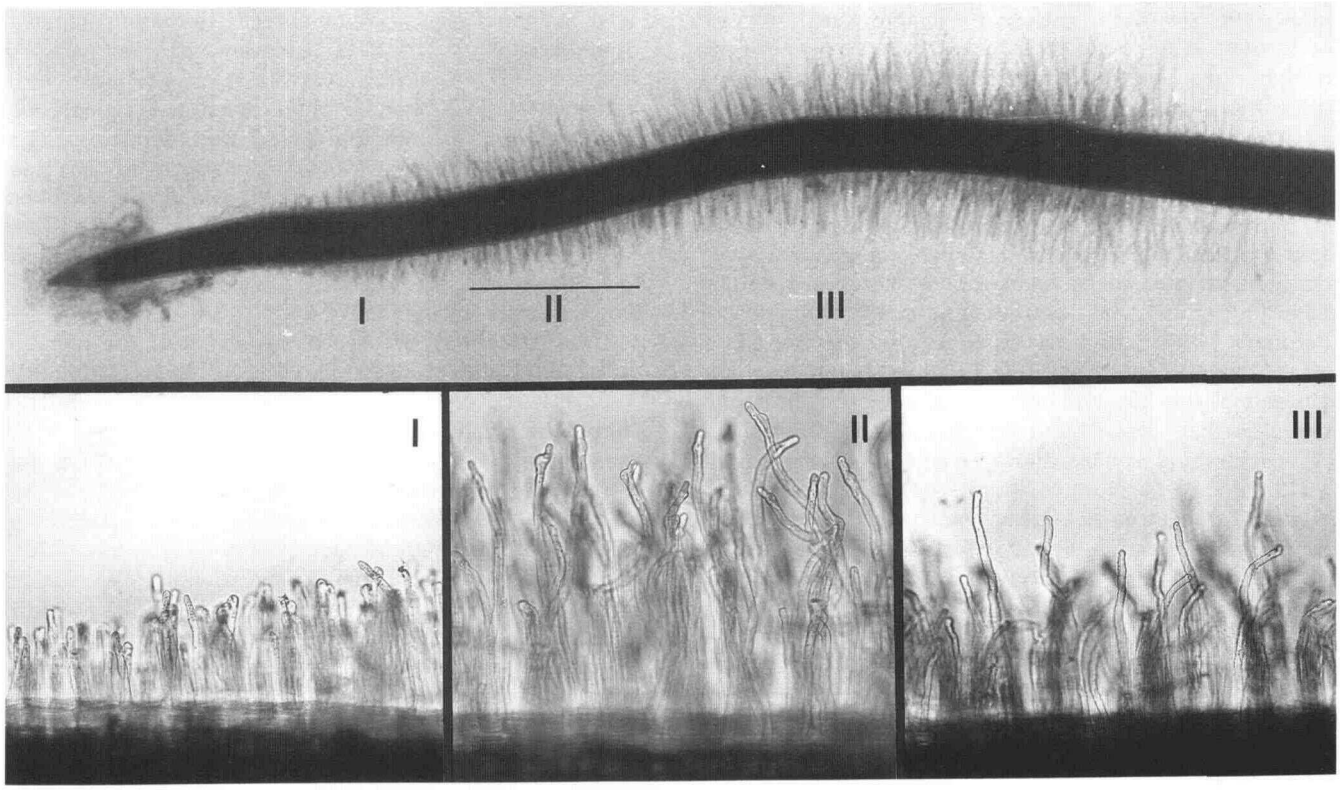

Figure 1. Root hair deformation on vetch roots. After applying NodRlv factor root hairs present in the susceptible zone (indicated by the line) deform after 3 h. The susceptible zone (II) of a vetch root encompasses young root hairs that have almost reached their mature state. This zone is about 2 mm long. Neither the young elongating root hairs (I) nor the older mature root hairs (III) are included in the susceptible zone.

The TLC system used for analysis consisted of Silica Gel 60 plates (Merck) with *n*-butanol:water:acetic acid (6:2:2) as mobile phase. A second system, used for the confirmation of co-migration with references, consisted of C_{18} reversed-phase TLC plates (Sigma) with acetonitrile:water (1:1) as a mobile phase. Both systems are described by Spaink et al. (1992).

The TLC plates were exposed for 2 d to a phosphor screen. The screen was scanned and the data were quantified using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA). As an alternative, autoradiograms of TLC plates containing tritiated compounds were scanned using a computing densitometer (Molecular Dynamics).

Accumulation of [^{3}H]NodRlv-V(C18:0,Ac) on Vetch Roots

Fåhraeus slides containing vetch plants were incubated with 10^{-9} M (2×10^5 cpm mL^{-1}) or 10^{-10} M (2×10^4 cpm mL^{-1}) [^{3}H]NodRlv-V(C18:0,Ac) for 2 and 10 min. Each experiment was performed two times using 10 Fåhraeus slides. Subsequently, the roots were washed five times with Fåhraeus medium containing 0.01% CHAPS, and the remaining radioactivity was determined after solubilizing the roots overnight in 0.5 mL of Soluene-350 (Packard) using liquid scintillation. The average percentage remaining on the roots was determined and the corresponding SE was calculated using the equation:

$$\mathrm{SE}(\bar{x}) = \frac{\sigma_{n-1}}{\sqrt{n}}.$$

Sectioning of *V. sativa* Roots

Fåhraeus slides containing vetch plants were incubated for 3 h with 2×10^5 cpm (10^{-9} M) [^{3}H]NodRlv-V(C18:0,Ac), after which the roots were washed five times with water containing 0.01% CHAPS. The roots were collected and fresh longitudinal sections (70 μm) were made using a fibratome (Bio-Rad). The sections were dried overnight on polylysine-coated slides. The slides were coated with Amersham LM-1 nuclear emulsion diluted 1:1 with 600 mM NH_4Ac and exposed for 2 weeks at −20°C. They were developed in Kodak D19 developer for 3 min and fixed in Kodak Fix. Sections were stained with 0.025% toluidine blue O for 5 min and mounted with DPX (BDH).

RESULTS

Vicia Root Hair Deformation Assay

V. sativa plants were grown in Fåhraeus slides as described in "Materials and Methods." Each Fåhraeus slide contained 1

mL of Fåhraeus medium and six plants that were grown for 2 d in these slides. Before the medium was exchanged with medium containing Nod factor, the root hairs were examined microscopically and Fåhraeus slides containing plants with deformed root hairs were discarded.

After addition of 10^{-9} M NodRlv-V(C18:4,Ac), root hairs deformed within 3 h and the deformed root hairs occurred in a narrow zone of the root. This susceptible zone was about 2 mm long, and as illustrated in Figure 1, encompassed young root hairs that had almost reached their mature size, but did not include either young elongating root hairs or old mature root hairs. The length of the root hairs in the deformation zone at time 0 increased by 10 to 20% over 24 h to reach the same size as the old root hairs that did not grow during this time. Root hair deformation was followed in time with a video camera and a typical timelapse series is shown in Figure 2. At time 0, 10^{-9} M NodRlv-V(C18:4,Ac) was added. Within 30 min cytoplasmic streaming was increased (data not shown), and within 1 h the root hair tip started to swell; this swelling was more pronounced after 1.5 h. After 2 h polar growth was initiated at the swollen root hair tip and after 3 h about 80% of the root hairs in the susceptible zone had the typical deformed appearance shown in Figure 2.

To study whether root hair deformation can be used as a semi-quantitative assay, we applied a series of concentrations ranging from 10^{-7} to 10^{-13} M NodRlv-V(C18:4,Ac) to the Fåhraeus slides and examined deformation after 3 h. Root hair deformation was scored blindly by two persons and at least two Fåhraeus slides were examined for each concentration. Concentrations of 10^{-7} to 10^{-11} M NodRlv-V(C18:4,Ac) had similar effects on root hair deformation, since at these concentrations about 80% of the hairs in the susceptible zone deformed. At the higher concentrations of this factor (10^{-7}, 10^{-8} M), a small percentage (1–5%) of the hairs in the susceptible zone branched. Since branching was a rather rare event and occurred only at high concentrations of Nod factor, we did not use this effect to score Nod factor activity. When plants were treated with 10^{-12} M NodRlv-V(C18:4,Ac), the percentage of deformed root hairs decreased to about 40%. At 10^{-13} M less than 10% of the root hairs deformed, and this level of deformation could not be distinguished from the level in control plants. We rated root hair deformation as 0, 1, or 2 according to the average percentage of root hairs that deformed 3 h after addition of Nod factor. A rating of 0 was given when 0 to 20% of the root hairs in the susceptible zone deformed, whereas ratings 1 and 2 were defined as 20 to 60% and >60% deformed root hairs, respectively. The reliability of this assay lies in the fact that over 90% of the plants tested with a certain concentration of Nod factor respond with an almost identical degree of deformation. So, by applying dilution series of Nod factors, this deformation assay can be used as a semiquantitative assay.

Using this assay, we tested the ability of the naturally occurring NodRlv factors [NodRlv-V(C18:4,Ac), NodRlv-IV(C18:4,Ac), NodRlv-V(C18:1,Ac), NodRlv-IV(C18:1,Ac)] and the radiolabeled NodRlv-V(C18:0,Ac). Each Nod factor was applied to vetch roots in a concentration range that varied from 10^{-7} to 10^{-13} M. The results in Table I show that C18:4- and C18:1-containing factors were able to induce root hair deformation (rating 2) at concentrations as low as 10^{-11} M, whereas the minimal concentration of NodRlv-V(C18:0,Ac) resulting in this rating was 10^{-10} M (Table I). This shows that the unsaturation of the acyl group is not a major determinant in the ability of NodRlv factors to induce root hair deformation.

Time of Nod Factor-Root Contact Required for Induction of Root Hair Deformation

As described above, swelling of root hairs tips was observed within 1 h after incubation with Nod factor, suggesting that only a short period of Nod factor-root contact is required to induce root hair deformation. The minimal period of Nod factor-root contact that leads to root hair deformation was determined by incubating vetch plants for 1, 2, 5, and 10 min with the lowest concentration of NodRlv-V(C18:4,Ac), NodRlv-IV(C18:4,Ac), or NodRlv-V(C18:0,Ac) that could induce deformation in a 3-h incubation period (Table I, rating 2). After incubation the Fåhraeus slides were washed five times with Fåhraeus medium containing 0.01% CHAPS and the plants were further incubated in Nod factor-free medium. Root hair deformation was scored 3 h later. Table II shows

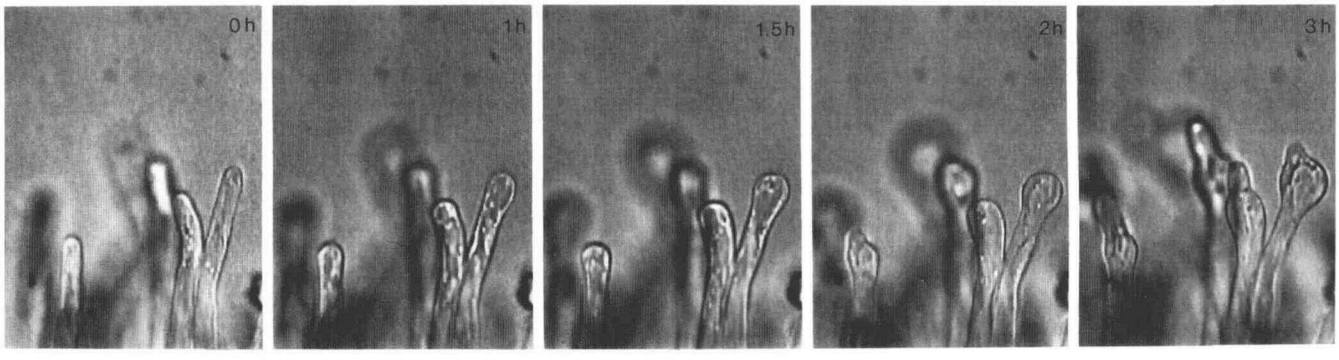

Figure 2. Root hair deformation followed in time. After applying 10^{-9} M NodRlv-V(C18:4,Ac) deformation was followed for 3 h using a video camera. After about 1 h the tips of the root hairs started to swell and this swelling was more pronounced after 1.5 h. After 2 h polar tip growth was initiated from the swollen root hair tips, and after 3 h the root hairs had a typical deformed appearance.

Table I. *The activity of Nod factors and Nod factor-derived compounds on root hair deformation*

Nod factors were applied to Fåhraeus slides containing vetch plants in a concentration range of 10^{-7} to 10^{-13} M. Deformation was examined as described in "Materials and Methods." Ratings 0, 1, and 2 correspond to 0 to 20, 20 to 60, and >60% deformed root hairs in the susceptible zone, respectively. Each experiment was performed at least five times using at least two Fåhraeus slides for each concentration of Nod factor.

Nod Factor	Concentration ($-\log_{10}$ M)						
	7	8	9	10	11	12	13
NodR1v-V(C18:4,Ac)	2	2	2	2	2	1	0
NodR1v-IV(C18:4,Ac)	2	2	2	2	2	1	0
NodR1v-V(C18:1,Ac)	2	2	2	2	2	1	0
NodR1v-IV(C18:1,Ac)	2	2	2	2	2	1	0
NodR1v-V(C18:0,Ac)	2	2	2	2	1	0	0
NodR1v-III(C18:4,Ac)	2	2	0	0	0	0	0
NodR1v-II(C18:4,Ac)	2	1	0	0	0	0	0

that the minimal incubation time with 10^{-11} M NodRlv-V(C18:4,Ac) and NodRlv-IV(C18:4,Ac) resulting in root hair deformation (rating 2) was 10 min, whereas a 5-min incubation period with 10^{-10} M NodRlv-V(C18:0,Ac) was sufficient to induce deformation (rating 2). Because more NodRlv-V(C18:0,Ac) was applied, a shorter time may be required for an amount of Nod factor able to induce complete root hair deformation to become associated with the root than for NodRlv-V(C18:4,Ac).

The amount of Nod factor remaining on the roots after washing was determined by incubating Fåhraeus slides for 2 or 10 min with either 10^{-9} M (2×10^5 cpm mL^{-1}) or 10^{-10} M (2×10^4 cpm mL^{-1}) [^{3}H]NodRlv-V(C18:0,Ac). Subsequently, the Fåhraeus slides were washed five times with Fåhraeus medium containing 0.01% CHAPS and the radioactivity bound to the roots was determined (see "Materials and Methods"). Table III shows that 1 to 2% of the originally applied radioactivity remained on the roots after washing.

Table II. *Minimal Nod factor incubation time for induction of root hair deformation*

Fåhraeus slides containing vetch plants were incubated with Nod factor for 1, 2, 5, or 10 min, after which the roots were washed with Fåhraeus medium containing 0.01% CHAPS and the plants were further incubated without Nod factor. As a control, Fåhraeus slides were incubated continuously in the presence Nod factor. Each experiment was performed at least three times using at least two Fåhraeus slides for each concentration of Nod factor.

Nod factor	Concentration	Incubation Time	Rating
	M	*min*	
NodR1v-V(C18:4,Ac)	10^{-11}	1	0
NodR1v-IV(C18:4,Ac)		2	0
		5	1
		10	2
		180	2
NodR1v-V(C18:0,Ac)	10^{-10}	1	0
		2	1
		5	2
		10	2
		180	2

Considering that only 1 to 2% of the applied Nod factor accumulated on the roots when a concentration of 10^{-10} M was applied and that a continuous incubation with a Nod factor concentration lower than 10^{-11} M did not lead to root hair deformation, it appears that the processes leading to deformation are initiated within 10 min.

Localization of Nod Factor Accumulation in Relation to Deformation

To determine whether Nod factors accumulate on a specific part of the roots, vetch plants were incubated with 10^{-9} M [^{3}H]NodRlv-V(C18:0,Ac) (2×10^5 cpm mL^{-1}) for 3 h. Fresh sections (70 μm) were made and exposed to a photographic emulsion. These studies showed that the label accumulated on the surface of the root; a relatively high amount was also detected on the root hairs (Fig. 3). Most of the radioactivity was found on the epidermal cells in the young part of the root, a region encompassing mature, susceptible, and actively growing root hairs. Less Nod factor accumulated on the old epidermal cells. Therefore, Nod factors preferentially accumulate on the young region of the root, but this accumulation is not restricted to the susceptible zone.

A detail of the susceptible zone containing deformed root

Table III. *Percentage of [^{3}H]NodR1v-V(C18:0,Ac) bound by vetch roots*

Ten Fåhraeus slides were incubated with 10^{-9} M (2×10^5 cpm mL^{-1}) or 10^{-10} M (2×10^4 cpm mL^{-1}) [^{3}H]NodR1v-V(C18:0,Ac) for 2 or 10 min, after which the roots were washed and the radioactivity remaining on the roots was determined. Calculation is as described in "Materials and Methods." Each experiment was performed two times using 10 Fåhraeus slides for each time point.

Concentration	Incubation Time	Percent Bound
M	*min*	
10^{-9}	2	1.1 ± 0.1
	10	1.7 ± 0.2
10^{-10}	2	0.9 ± 0.1
	10	1.8 ± 0.2

hairs shows that the Nod factor is mostly evenly distributed over the root hairs (Fig. 4). However, on some deformed root hairs label is present in a tonsure-like pattern around the tip of the root hair (indicated by arrowheads), whereas the newly formed tip itself does not contain any label. Plasmolyzed root hairs show that Nod factor is located in the cell membrane (indicated by arrows).

The Fate of Nod Factors during Root Hair Deformation

To determine the stability of Nod factors during the period in which root hair deformation takes place, we incubated vetch plants with 6×10^{-7} M (15,000 cpm mL^{-1}) [^{14}C]NodRlv-V(C18:4,Ac) or [^{14}C]NodRlv-IV(C18:4,Ac). After 10, 60, and 180 min the medium of three Fåhraeus slides was collected and the roots were washed five times with Fåhraeus medium. The medium and the roots were extracted with *n*-butanol. Both the water phase and the *n*-butanol phase were analyzed by TLC. Since we detected hardly any radiolabeled compounds in the water phase, these data are not shown. It is likely that GlcNAc would be present in the water phase. Therefore, we believe that the GlcNAc molecules that were cleaved off were quickly metabolized or immobilized. Alternatively, our TLC system may not be suitable for analyzing these sugars.

The results of a typical experiment with [^{14}C]NodRlv-V(C18:4,Ac) are shown in Figure 5. Three new compounds (B, C, and D) were produced in the medium and on the roots. By comparing the R_F values with those of references, using two different TLC systems, it was shown that the R_F values of spots B, C, and D corresponded with NodRlv factors with a shortened glucosamine backbone, a tetramer ([^{14}C]NodRlv-IV(C18:4,Ac)), a trimer ([^{14}C]NodRlv-III(C18:4,Ac)), and a dimer ([^{14}C]NodRlv-II(C18:4,Ac)), respectively. When NodRlv-IV(C18:4,Ac) was applied, C (trimer) and D (dimer) were formed. This suggests that Nod factors are degraded by chitinases.

The amount of radioactivity of the different compounds was determined with a PhosphorImager and expressed as a percentage of the total amount of radioactivity recovered from the medium and from the roots at each time point. We performed three experiments in which we compared the fate of the pentamer [NodRlv-V(C18:4,Ac)] and the tetramer [NodRlv-IV(C18:4,Ac)]. These experiments gave similar results; results from a representative experiment are shown in Figure 6.

In the medium 19% of the pentamer was degraded after 3 h and the major breakdown product was the tetramer (13%). The trimer and dimer were present in equal amounts (3%). In contrast, only 6% of the tetramer was degraded into trimer (2%) and dimer (4%). Thus, the pentamer was degraded more rapidly than the tetramer.

Nod factors accumulated on the roots during incubation, and after 3 h about 15% of the originally applied radioactivity was associated with the roots and their Nod factors were rapidly degraded. Within 10 min one-half of the pentamer was already degraded. However, degradation of the tetramer during this time was much slower. When the pentamer was applied to the roots, its degradation product, the tetramer,

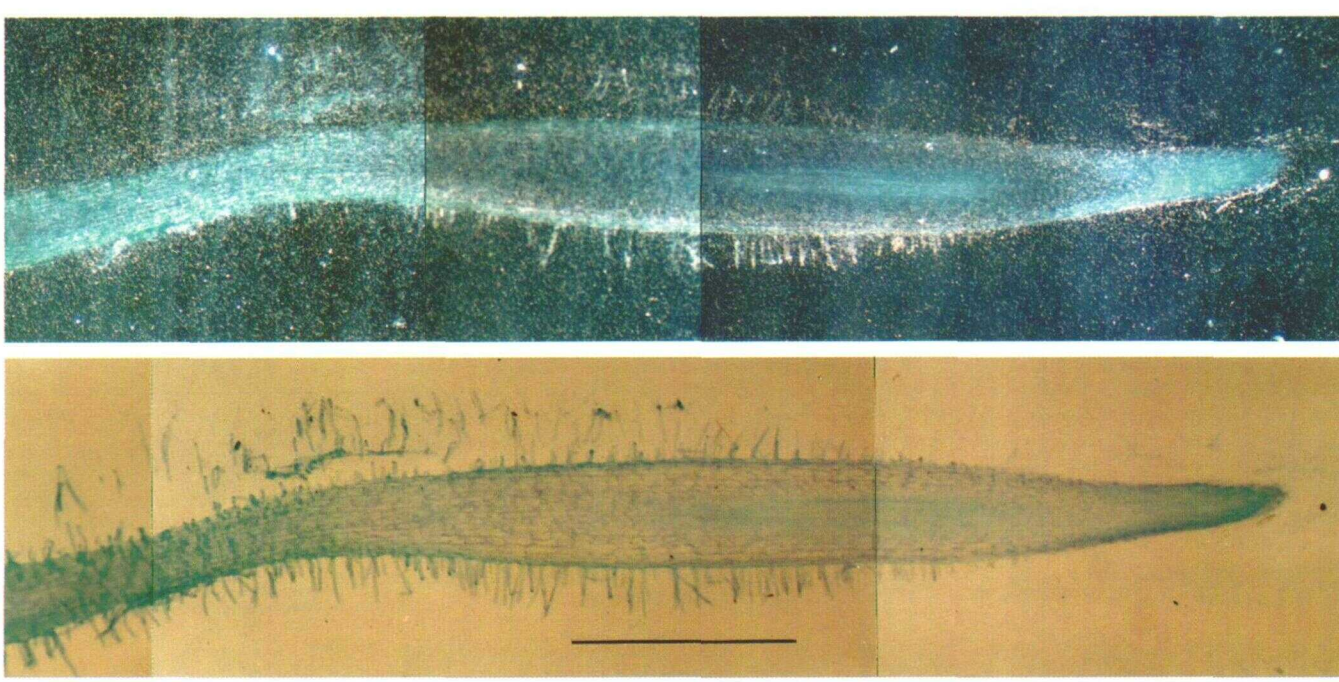

Figure 3. Localization of [^{3}H]NodRlv-V(C18:0,Ac) on vetch roots. Fresh longitudinal sections were made of roots incubated for 3 h in the presence of 10^{-9} M (2×10^{5} cpm mL^{-1}) [^{3}H]NodRlv-V(C18:0,Ac) after washing the roots five times with Fåhraeus medium containing 0.01% CHAPS. The dark-field and bright-field pictures show that the label is present on the epidermal cells (including root hairs) of the root, but this accumulation is not restricted to the susceptible zone (indicated by the line).

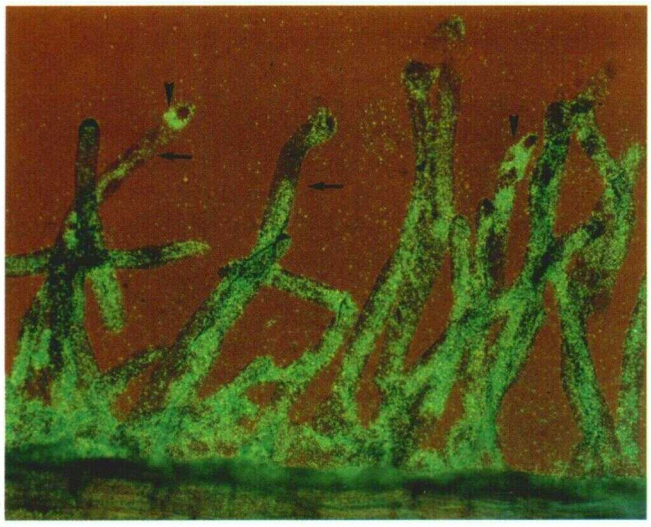

Figure 4. Detail of Figure 3 using epipolarization microscopy. Some deformed root hairs contained label in a ring around the swollen root hair tip (indicated by arrowheads) but not on the polar outgrowth formed from this tip. Other root hairs that are plasmolyzed contain label in the cell membrane (indicated by arrows).

was always found at higher levels than the pentamer (except after 10 min). These observations confirm that the tetramer is more stable than the pentamer. The ratio of dimer to trimer was similar to that in the medium. When the pentamer was applied, a 1:1 ratio of dimer to trimer was found on the roots (approximately 3% of each after 3 h), whereas the dimer and trimer were found in a ratio of 2:1 (approximately 4% versus approximately 2% after 3 h) after incubation with the tetramer.

In addition to the chitinase degradation products, occasionally a product was formed that co-migrated with palmic acid. We assume that this was the released C18:4 fatty acid (data not shown).

Root hair deformation was induced by NodRlv factor at concentrations as low as 10^{-11} M. The relatively high concentration of 6×10^{-7} M [^{14}C]NodRlv-V(C18:4,Ac) used in these studies might not be physiological. Therefore, we also examined degradation of [^{3}H]NodRlv-V(C18:0,Ac), which had a higher specific activity (200 Ci mmol^{-1}). Fåhraeus slides containing vetch plants were incubated with 10^{-9} M (2×10^{5} cpm mL^{-1}) NodRlv-V(C18:0,Ac), and degradation of this factor was studied as described above. Because a tritiated molecule was used, the radiograms were quantified with a computing densitometer. The rate of degradation of NodRlv-V(C18:0,Ac) was similar to that of NodRlv-V(C18:4,Ac), and the percentage of the pentamer that bound to the roots was comparable. The tetramer was also the major breakdown product. However, the 5:1 ratio of dimer to trimer found on the roots differed from the 1:1 ratio observed during NodRlv-V(C18:4,Ac) incubation.

To determine whether the trimer and the dimer are able to induce deformation, these compounds were applied to vetch plants in concentrations varying from 10^{-7} to 10^{-13} M. As described above, the pentamer and tetramer induced deformation at concentrations as low as 10^{-11} M. However, as shown in Table I, the minimal concentration of trimer and dimer that induced deformation (rating 2) was much higher, 10^{-8} and 10^{-7} M, respectively, indicating that a GlcN backbone of four or five sugar residues is optimal for induction of deformation. Therefore, it is unlikely that degradation, which leads to the formation of Nod factors at least 1000-fold less active than the originally applied Nod factors, is involved in the induction of root hair deformation.

Nod Factor Degradation in Relation to Biological Activity

To determine whether the degradation of Nod factors controls where and when Nod factors are active, we studied the correlation between the ability to respond to Nod factors by root hair deformation and the ability to degrade Nod factors. Since root hair deformation occurs only in the susceptible zone of the root, we studied whether Nod factors are degraded only in the zone with mature root hairs.

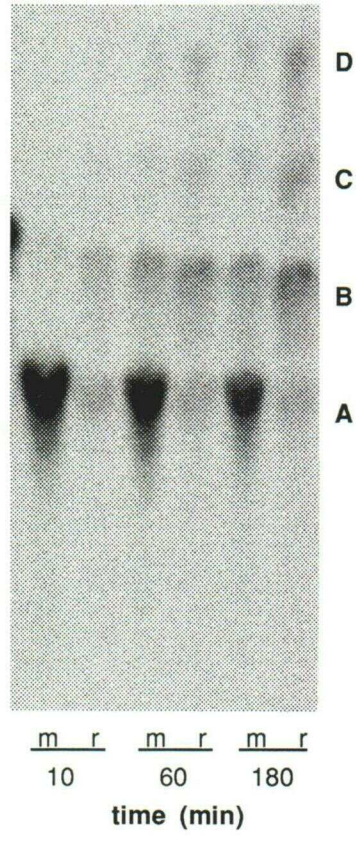

Figure 5. A typical TLC analysis of *n*-butanol-extracted medium (m) and roots (r). At 10, 60, and 180 min after applying 6×10^{-7} M (15,000 cpm mL^{-1}) [^{14}C]NodRlv-V(C18:4,Ac) to Fåhraeus slides, *n*-butanol extracts of the medium and roots of three Fåhraeus slides were made, and one-third and all of the extract was spotted on TLC, respectively. The TLC system used consisted of Silica Gel 60 plates with *n*-butanol:water:acetic acid (6:2:2) as a mobile phase. The positions of the reference compounds A ([^{14}C]NodRlv-V(C18:4,Ac)), B ([^{14}C]NodRlv-IV(C18:4,Ac)), C ([^{14}C]NodRlv-III(C18:4,Ac)), and D ([^{14}C]NodRlv-II(C18:4,Ac)) are indicated.

Sixty Fåhraeus slides containing vetch plants were incubated for 3 h with 5×10^{-9} M (10^6 cpm mL^{-1}) [^{3}H]NodRlv-V(C18:0,Ac), and after washing the slides the susceptible zone and the mature zone were collected. *n*-Butanol extraction, TLC analysis, and quantification were performed as described in "Materials and Methods." As is shown in Figure 7, Nod factors are degraded in a similar manner in the mature and susceptible zone, indicating that degradation of Nod factors does not control the susceptibility of regions of the root. Also shown is the ratio of 5:1 for the dimer and trimer as described above.

The nonhost alfalfa does not respond to Nod factors lacking a sulfate group on the reducing sugar (Lerouge et al., 1990). Therefore, we compared the degradation of NodRlv-V(C18:4,Ac) on vetch and alfalfa roots. Alfalfa plants were germinated and grown in Fåhraeus slides as described for vetch and incubated for 3 h with 6×10^{-7} M (15,000 cpm mL^{-1}) [^{14}C]NodRlv-V(C18:4,Ac). *n*-Butanol extraction, TLC analysis, and quantification were performed as described for vetch. As shown in Figure 8, a similar amount of Nod factor is degraded on vetch and alfalfa after a 3-h incubation period. Therefore, the inability of alfalfa to respond to NodRlv factors is not caused by an enhanced degradation of NodRlv factors on the root system of this nonhost plant.

The Effect of NH_4NO_3 on Root Hair Deformation and Nod Factor Degradation

Nitrate has been known for a long time to inhibit root hair deformation on legumes inoculated with *Rhizobium* (Thornton, 1936). Root hairs of vetch plants grown in the presence of 10 mM NH_4NO_3 are not able to deform after incubation with up to 10^{-7} M NodRlv-V(C18:4,Ac) (data not shown). To determine the time required for NH_4NO_3 to cause this block in deformation, vetch plants grown in Fåhraeus slides were preincubated with 10 mM NH_4NO_3 for 0 to 48 h. Root hair deformation was rated 3 h after applying 10^{-9} M NodRlv-V(C18:4,Ac). Table IV shows that only a preincubation period of 36 h or longer completely blocked root hair deformation.

Since the root keeps growing during this 36 h, we tested whether root hairs formed before NH_4NO_3 addition are affected in deformation ability. The root systems were examined at the time of NH_4NO_3 addition (time 0) and 36 h later when Nod factor was applied. The position of the susceptible zone of the roots was marked on the coverslip of the Fåhraeus slide at both time points. This experiment showed that the susceptible zones at 0 and 36 h do not overlap, since at 36 h the new susceptible zone was located in the region of the root that did not yet contain root hairs at time 0. This means that the old and the new susceptible zones were about 2 mm apart. So, a complete block of deformation was obtained only when a root hair was formed in the presence of NH_4NO_3.

To determine whether the inhibition of root hair deformation by NH_4NO_3 is reversible, we grew the vetch plants in the presence of 10 mM NH_4NO_3 for 48 h, after which the Fåhraeus slides were washed five times with Fåhraeus medium without NH_4NO_3. Subsequently, the plants were grown for 0 to 48 h in the absence of NH_4NO_3 before 10^{-9} M NodRlv-V(C18:4,Ac) was applied. As shown in Table V, root hair deformation can first be induced 36 h after transfer to an NH_4NO_3-free medium. This suggests that root hairs formed in the presence of NH_4NO_3 do not regain the ability to deform.

We tested whether the presence of NH_4NO_3 in the medium affects Nod factor degradation. Vetch plants were grown in Fåhraeus slides with Fåhraeus medium containing 10 mM NH_4NO_3 and incubated for 3 h with 6×10^{-7} M (15,000 cpm mL^{-1}) NodRlv-V(C18:4,Ac). Figure 8 shows that NodRlv-V(C18:4,Ac) is degraded in a similar way on roots of control vetch plants and on roots of plants grown in the presence of 10 mM NH_4NO_3. Hence, it is very unlikely that the inhibitory

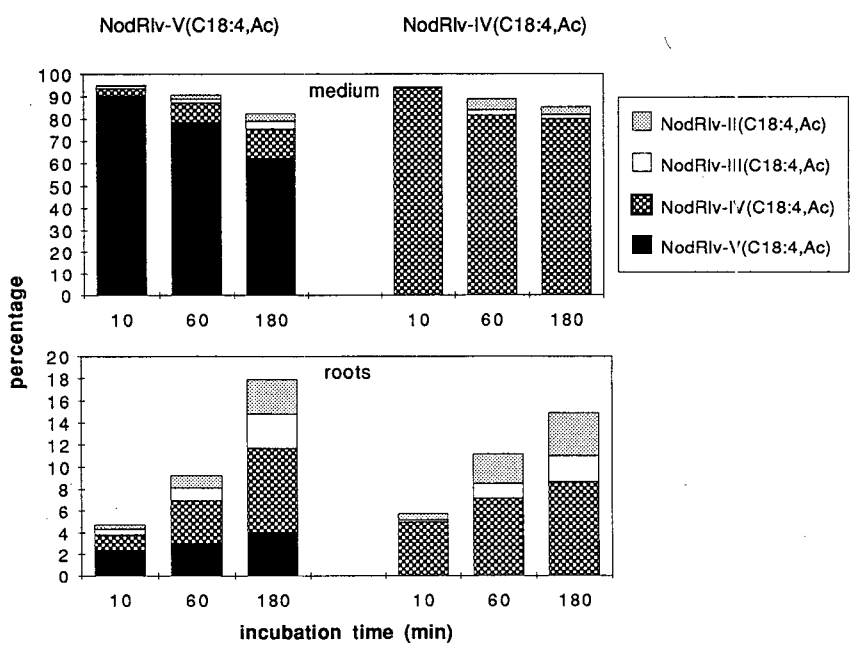

Figure 6. Degradation products present in the medium and on roots contained on Fåhraeus slides after incubation with [^{14}C]NodRlv-V(C18:4,Ac) and [^{14}C]NodRlv-IV(C18:4,Ac). On roots the pentamer was rapidly converted mainly into tetramer, which is always present at higher levels than the pentamer (except at 10 min). In both medium and roots the ratio of dimer to trimer present after incubation with the pentamer was 1:1, whereas after incubation with the tetramer the ratio was 2:1.

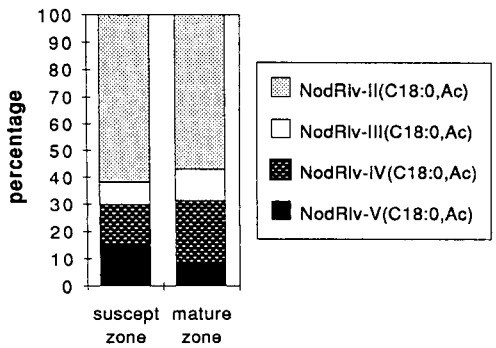

Figure 7. Comparison of degradation of [^{3}H]NodRlv-V(C18:0,Ac) in the susceptible zone and the mature zone. Fåhraeus slides were incubated with [^{3}H]NodRlv-V(C18:0,Ac) (2×10^{-9} M, 2×10^5 cpm mL^{-1}) for 3 h. The experiment was performed in duplicate and the amount of radioactivity was considered 100% for each zone. In both zones about equal degradation took place.

effect of NH_4NO_3 on root hair deformation is caused by an increased Nod factor degradation.

DISCUSSION

We show here that the root hair deformation assay described is fast and simple and can be used as a semiquantitative assay to determine the activity of Nod factors. Furthermore, root hair deformation can be followed microscopically. Nod factor-induced root hair deformation is a rapid response of the plant. The first deformation occurs within 1 h after Nod factor application, whereas an increased cytoplasmic streaming occurs within 30 min (data not shown). In most other studies, root hair deformation was first scored several days after Nod factor application (Lerouge et al., 1990; Spaink et al., 1991; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; Mergaert et al., 1993). Therefore, it is not clear whether such studies provide an accurate comparison of the abilities of different Nod factors to induce deformation. We also used this deformation assay to study alfalfa root hair deformation and showed that NodRm factors induce clear deformation within 3 h (data not shown). Since clover root hair deformation is also induced within 3 h (Bhuvaneswari and Solheim, 1985), it is likely that for several small legumes this root hair deformation assay will prove to be a rapid and simple assay with which to study the activity of Nod factors.

As described, root hair deformation is induced by both C18:4- and C18:1-containing NodRlv factors, whereas cortical cell divisions and preinfection thread formation can be induced only by NodRlv factors containing a C18:4 acyl group (Spaink et al., 1991; Van Brussel et al., 1992). This shows that the deformation assay is less specific with regard to the structure of Nod factors. Consequently, the root hair deformation assay cannot be used to study the interaction between Nod factor and legume roots that requires such a high structure specificity.

Monitoring root hair deformation with a video camera showed that the existing tips of root hairs swell before polar growth is induced. A similar swelling of root hair tips can be induced by a treatment with hydrolytic enzymes (Cocking, 1985). Therefore, it is possible that Nod factors induce tip swelling by targeting hydrolytic enzymes to the tips of root hairs. The tip swelling is followed by new polar growth from the swollen root hair tip. During the formation of root hairs, the induction of tip growth is preceded by a local hydrolysis of the epidermal cell wall (Dazzo et al., 1987; Bakhuizen, 1988). Thus, the sequence of events during root hair formation and deformation are similar. Therefore, we postulate that the mechanism of root hair deformation is derived from the process controlling root hair development. This hypothesis is supported by the ability of Nod factors to stimulate root hair development (Roche et al., 1991a).

Root hair deformation is induced only in a small zone of the root containing root hairs that have almost stopped growing. However, in *Medicago* ENOD12 gene expression and root hair membrane depolarization are induced in a broader zone, including mature and young root hairs (Ehrhardt et al., 1992; Pichon et al., 1993). These results show that this entire zone is susceptible to Nod factor, whereas only in a limited part of this zone do root hairs deform. This again suggests that only at a specific stage of development are root hairs able to deform.

Nitrate has been shown to be an effective inhibitor of root hair deformation (Thornton, 1936). Here we show that root hairs that developed in the presence of NH_4NO_3 are unable

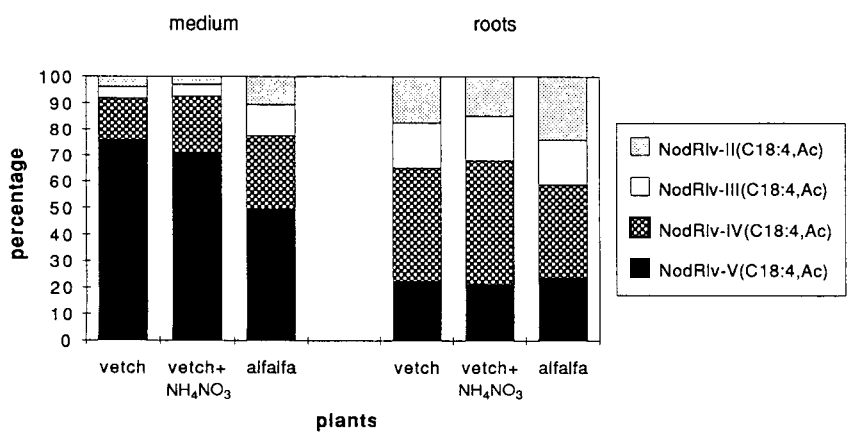

Figure 8. Comparison of degradation with vetch, vetch grown in the presence of 10 mM NH_4NO_3, and alfalfa. Fåhraeus slides were incubated with [^{14}C[NodRlv-V(C18:4,Ac) (6×10^{-7} M, 15,000 cpm mL^{-1}) for 3 h. Each experiment was performed at least two times and the results of these experiments were similar. The amount of radioactivity was considered 100% for the medium and the roots. The degradation pattern and rate in both medium and roots did not differ significantly from that observed for the control vetch plants.

Table IV. *Minimal NH_4NO_3 preincubation time required to inhibit root hair deformation*

Fåhraeus slides were preincubated with 10 mM NH_4NO_3 for 0 to 48 h. Deformation was examined 3 h after applying 10^{-9} M NodR1v-V(C18:4,Ac). Each experiment was performed at least five times using at least two Fåhraeus slides.

Incubation time (h)	0	3	6	12	24	36	48
Rating	2	2	2	2	1	0	0

to deform, whereas NH_4NO_3 could not block deformation in root hairs that developed in the absence of NH_4NO_3. This suggests that NH_4NO_3 blocks the synthesis of a component(s) involved in the perception or transduction of Nod factors. Alternatively, NH_4NO_3 may alter root hair development so that root hairs can no longer deform.

Studies on the fate of Nod factors during incubation with vetch roots showed a rapid degradation of these factors, most likely by chitinases. This chitinase activity is associated mainly with the roots, since the relative amount of breakdown products on the roots is markedly higher than in the medium. Incubation with 10^{-9} M [^{3}H]NodRlv-V(C18:0,Ac) results in a 5:1 ratio of dimer to trimer. But because dimers are the terminal products formed after chitinase digestion of Nod factors, we assume that relatively more dimer is produced because the substrate concentration is markedly lower and the reaction is enzyme limited.

Because Nod factors are rapidly degraded, it can be questioned whether the rate of Nod factor degradation determines which part of the root system will respond to Nod factors. We showed that after 3 h the degradation of Nod factors is similar in the susceptible zone and the zone containing mature root hairs, and consequently, that degradation of Nod factors does not appear to be a major determinant of the susceptibility to Nod factors. This does not exclude the possibility of a differential degradation at an earlier time point, e.g. at 10 min. However, since the amount of degradation products is markedly lower at 10 min, it was not possible to perform this experiment. Furthermore, we showed that 3 h after application the degradation of NodRlv-V(C18:4,Ac) by the nonhost alfalfa is similar to the degradation by vetch. Moreover, we showed that NH_4NO_3, which completely blocks deformation, had no effect on Nod factor breakdown. Since our experiments did not show a correlation between Nod factor degradation and the potential to deform root hairs, we conclude that degradation cannot account for the differential response of root hair deformation. The fact that the degradation products can induce root hair deformation only at 1000-fold higher concentrations indicates that Nod factor degradation is not of importance in deformation.

A 10-min period of Nod factor-root contact appears to be sufficient to induce root hair deformation, but when the Nod factor-treated roots were extensively washed, about 2% of the originally applied Nod factor remained on the root system. Therefore, it cannot stated with certainty that this small quantity of Nod factor does not induce root hair deformation at a later stage. However, since we used the minimal concentration of Nod factor that can efficiently induce deformation and since Nod factors are rapidly degraded on the root surface, we think it is most likely that within 10 min Nod factor perception occurs and signal transduction has been initiated. This conclusion is consistent with the observation of Ehrhardt et al. (1992), who showed that root hair membrane depolarization is induced within 10 min. In several animal systems it has been shown that a short period of ligand-receptor recognition is sufficient to set a signal-transduction process in motion (Chao, 1992; Lefkowitz, 1993). Therefore, the fact that Nod factors are active at concentrations as low as 10^{-11} M and our observation that a 10-min period is sufficient to start root hair deformation support the hypothesis that a receptor is involved in Nod factor recognition.

If a receptor is involved, it is unknown which molecule would bind to such a putative receptor. Is this the unmodified Nod factor or are Nod factor-derived molecules recognized? Degradation of Nod factors occurs within 10 min; hence, we cannot be certain that NodRlv-III(C18:4,Ac) or NodRlv-II(C18:4,Ac) is not recognized by a receptor. However, since the trimer and dimer have strongly reduced abilities to elicit deformation, it is not very likely that these compounds play a role in the induction of root hair deformation.

A comparison of the degradation rate of NodRlv-V(C18:4,Ac) and NodRlv-IV(C18:4,Ac) showed that the tetramer is the more stable of the two. On vetch roots the pentamer was degraded rapidly, and within 10 min after application it encompassed less than 50% of the NodRlv compounds present on the root; at all time points tested (except at 10 min) the tetramer was present at higher quantities than the pentamer. The latter observation supports the conclusion that the tetramer is more stable than the pentamer.

Our studies are in agreement with those of Schultze et al. (1993), who showed that NodRm-V(C16:2,S) is degraded faster than NodRm-IV(C16:2,S). Schultze et al. (1993) showed that the NodRm-pentamer is 100-fold less active in inducing *Medicago* root hair deformation than the NodRm-tetramer. This is in contrast to the equal potential of NodRlv-

Table V. *Reversibility after NH_4NO_3 treatment*

Fåhraeus slides were preincubated for 48 h in the presence of 10 mM NH_4NO_3. Subsequently, the slides were washed and the plants were further incubated in the absence of NH_4NO_3 for 0 to 48 h, after which 10^{-9} M NodR1v-V(C18:4,Ac) was added and deformation was examined 3 h later. Each experiment was performed at least five times using at least two Fåhraeus slides.

Time of NH_4NO_3 absence (h)	0	3	6	12	24	36	48
Rating	0	0	0	0	1	2	2

pentamer and NodRlv-tetramer to induce root hair deformation in vetch (Spaink et al., 1991). We showed that NodRlv-pentamer is rapidly converted into the tetramer, which is equally active on vetch roots. However, removal of the terminal sugar of the sulfated NodRm-pentamer results in the nonsulfated tetramer, a compound inactive on alfalfa roots. Therefore, it is quite possible that vetch and alfalfa Nod factor receptors preferably recognize NodRlv-IV(C18:4,Ac) and NodRm-IV(C16:2,Ac,S), respectively.

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