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Nod factors and *tri*-iodobenzoic acid stimulate mycorrhizal colonization and affect carbohydrate partitioning in mycorrhizal roots of *Lablab purpureus*

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SUMMARY

Roots of *Lablab purpureus* (L.) Sweet were treated with *tri*-iodobenzoic acid (TIBA), kinetin or with nodulation factors (Nod factors) purified from *Rhizobium* sp. NGR234 and grown in the presence of a mycorrhizal inoculum (*Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe. Colonization by the mycorrhizal fungus was increased from < 30 % to *c.* 65 % of root length when roots were treated with these growth regulators. Moreover, treatment of mycorrhizal *L. purpureus* roots with Nod factors or TIBA strongly induced sporocarp formation of *Glomus mosseae*. In parallel, the pool size of the fungal disaccharide trehalose was significantly affected in roots treated with TIBA and Nod factors alone, and with TIBA combined with all effectors, and increased from 0·06 mg g^{-1} d. wt in control roots to up to 1·7 mg g^{-1} d. wt (TIBA + kinetin). Conversely, the sucrose pool decreased from 5 % d. wt to less than a half in roots treated with Nod factors. Activities of trehalase were significantly enhanced in mycorrhizal roots by the treatment with Nod factors or TIBA. When Nod factors and TIBA were added in combination, these activities were strongly enhanced suggesting synergism between these growth regulators.

Key words: Arbuscular mycorrhiza, *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe, morphogenesis, nodulation, phytohormones, *Rhizobium* sp. NGR234 symbiosis, trehalase.

INTRODUCTION

Balances between plant hormones are not only crucial for normal morphogenesis, but also for the establishment of symbioses like the nodulation of legumes (Hirsch & Fang, 1994). The auxin transport inhibitor tri-iodobenzoic acid (TIBA) elicits nodule-like structures and induces early nodulins (Hirsch *et al.*, 1989), perhaps by increasing endogenous cytokinin levels (Dehio & de Bruijn, 1992; Cooper & Long, 1994). Plant hormones, and possibly molecules related to Nod factors (a family of chitin-based, lipo-oligosaccharides; Fellay *et al.* (1995); Dénarié, Debellé & Promé (1996)), might also be involved in the establishment of mycorrhizal associations. Increased levels of cytokinin (Allen, Moore & Christensen, 1980; Dixon, Garrett & Cox, 1988; Thiagarajan & Ahmad, 1994) and of auxin (Ludwig-Müller *et al.*, 1997) in mycorrhizal roots have been reported. Recently, we have shown that rhizobial Nod factors stimulate the mycorrhizal colonization in soybean roots (Xie *et al.*, 1995).

Here, we examine the effects of Nod factors, TIBA, and kinetin on colonization of roots of *Lablab purpureus* by the mycorrhizal fungus *Glomus mosseae*. Carbohydrate partitioning, particularly of the fungal disaccharide trehalose (Müller, Boller & Wiemken, 1995*a*) was also studied. *Lablab* was chosen since this legume reacted strongly to growth regulators in preliminary experiments. Furthermore, we show effects on trehalase, an activity strongly enhanced in nodules (Müller *et al.*, 1995*a*) and inducible in sterile roots by auxin (Müller, Boller & Wiemken, 1995*b*). The Nod factors used in the experiments came from *Rhizobium* sp. NGR234, a strain which effectively nodulates *Lablab* and a variety of other legumes (Pueppke & Broughton, unpublished).

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Abbreviations: Nod factors, nodulation factors; TIBA, *Tri*-iodobenzoic acid.

MATERIALS AND METHODS

Growth of plant material

Seeds of *Lablab purpureus* (L.) Sweet were surface-sterilized by immersion in H_2O_2 (30% (w/v)) for 20 min followed by washing with sterile tap-water. After growth for 3 d at 27 °C in the dark on plates containing 1% (w/v) agar in Broughton & Dilworth (B&D) nutrient solution (Broughton & John, 1979) amended with 2·5 mM KNO_3, they were planted individually into Magenta jars (Sigma, Buchs, Switzerland) containing mycorrhizal inoculum (roots, sporocarps in sand/loam), sand, and loam in the ratio 1:1:1 (v/v/v) thus saturating the substrate with mycorrhizal propagules. Sand and loam were autoclaved before use. The B&D-nutrient solution (0·2 l in the lower compartment of the jar) was supplemented with the effectors to be tested. Plants were grown in a phytotron (14-h day at 300 μmol photons s^{-1} m^{-1} and 26 °C, 10 h night at 20 °C). Sterile roots were harvested, immediately frozen and held at −20 °C for further use. For nodulation experiments, seedlings were prepared as described above, planted into Leonard jars, inoculated with *Rhizobium* sp. NGR 234 and cultivated as described (Müller *et al.*, 1995*b*).

Mycorrhizal roots were harvested after 4–5 wk of growth, thoroughly washed, immediately frozen and stored at −20 °C. Mycorrhizal colonization, expressed as the percentage of root length containing mycorrhizal structures, was estimated as described earlier (Xie *et al.*, 1995). Sporocarps formed by extraradical mycelium and attached to the the rhizoplane were counted in a fraction of the root system (25% of total roots) and extrapolated.

Plant growth regulators

Acetylated and sulphated Nod factors (Fig. 1) from *Rhizobium* NGR234 were purified according to Price *et al.* (1992). Tri-iodobenzoic acid (TIBA) and kinetin were obtained from Fluka (Buchs, Switzerland). These growth regulators were added to the nutrient solution in the lower part of the Magenta jar (0·1 μM for Nod factors and kinetin; 50 μM for TIBA) 1 wk after planting (one addition per growth period).

Analysis of non-structural carbohydrates

Soluble carbohydrates were analysed by gas chromatography; starch was determined enzymatically according to previously published protocols (Müller *et al.*, 1995*b*).

Enzyme activities

Frozen roots were ground in MES (K^+) buffer (0·1 M, pH 6·3) containing EDTA and PMS (2 mM each). The buffer:sample ratio was 1 ml g^{-1} f. wt. Crude extracts were centrifuged at 20000 ***g*** for 10 min. Trehalase was assayed according to Müller *et al.* (1992) by incubating enzyme aliquots in MES (K^+) buffer (pH 6·3, 50 mM) containing 10 mM trehalose at 37 °C for 15– 60 min. Reactions were terminated by boiling and the glucose generated was determined using glucose oxidase-peroxidase (Boehringer Mannheim GmbH, Mannheim, Germany). Substrate and enzyme blanks were incubated in parallel.

Nitrogenase activity was measured using the acetylene reduction assay as described earlier (Müller *et al.*, 1995*b*).

Statistics

Analyses of variance and Fisher's protected LSD-tests were performed using the software SuperANOVA (Abacus, Berkeley, CA, USA); *t*-tests were performed according to Student or to Welch (in the case of heteroscedasticity).

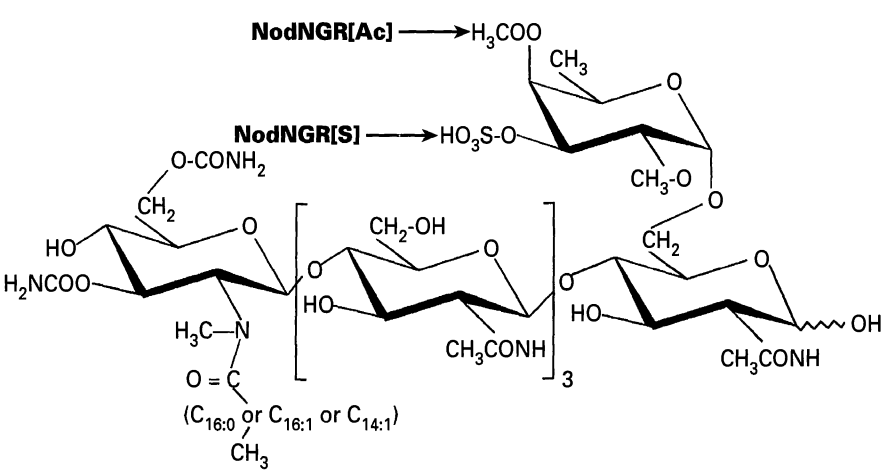

Figure 1. Structure of Nod factors purified from *Rhizobium* sp. NGR 234. The fucose residue was either sulphated (NodNGR[S]) or acetylated (NodNGR[Ac]).

RESULTS

Effects of TIBA on the establishment of symbioses

In many legumes, TIBA induces the formation of 'thick, short roots' (Hirsch *et al.*, 1989). To test whether TIBA affected the formation of functional root nodules, seedlings were inoculated with *Rhizobium* NGR234 and grown until the onset of fruiting. TIBA interfered with shoot growth reducing the shoot biomass significantly at harvest (from 7 to 2·5 g f. wt mean), but substantially increased nodule number (from 89 to 202 mean). However, both nodule biomass and nitrogenase activity per nodule f. wt were drastically reduced (Table 1).

The effect of TIBA on mycorrhizal infection was also examined. To do this, the plants were inoculated with *Glomus mosseae* in a similar culture system, but with a different soil composition and under non-sterile conditions, in the presence or absence of TIBA. Interestingly, TIBA strongly stimulated mycorrhizal colonization increasing the average infection rate from 58 to 95 % of root length despite the inhibitory effects on shoot growth (Table 2). It is, however, noteworthy that TIBA-treated roots were much shorter than untreated roots: the length: mass ratio was *c.* $2{\cdot}2 \pm 0{\cdot}4$ m g^{-1} f. wt for normal roots, and $0{\cdot}9 \pm 0{\cdot}2^{-1}$ m g^{-1} f. wt (means ± SD for three independent plants) for TIBA-treated roots. Therefore, total fungal biomass was clearly higher in control than in TIBA-treated roots.

Nod factors and TIBA induce sporocarp formation on mycorrhizal roots

To see whether Nod factors and kinetin could affect mycorrhizal colonization in the same way as TIBA, but without altering the root morphology, *L. purpureus* plants were cultivated in the presence of a mycorrhizal inoculum and treated with TIBA, kinetin and Nod factors, alone or in combination. The degree of mycorrhizal colonization of untreated *L. purpureus* roots was *c.* 30 % in this experiment. Significant increases up to 60 % were observed following treatment with either TIBA or Nod factors alone. A combination of the acetylated Nod factor and TIBA had about the same effect as the Nod factor alone. These were not mere growth effects, since root biomass was significantly higher in plants treated with Nod factors as compared with TIBA, although the degrees of mycorrhizal infection were about the same. Moreover, treatments with Nod factors, TIBA and kinetin strongly stimulated sporocarp formation from *c.* 10 per plant to > 100 (Table 3).

Table 2. *Effects of TIBA on growth and mycorrhizal colonization of* Lablab purpureus

Treatment	Shoot f. wt (g)	Mycorrhizal colonization (% of root length)
Control	$11{\cdot}5 \pm 1{\cdot}1$	$58 \pm 4{\cdot}7$
TIBA	$3{\cdot}3 \pm 0{\cdot}5$	$95 \pm 1{\cdot}5$
α	< 0·01	< 0·02

Plants were inoculated with *Glomus mosseae*, grown under phytotron conditions and harvested at the onset of fruiting of the control plants. The values given are means ± SE for three independent plants. The data were tested for significance using *t*-tests and the error probability α is indicated.

Effects on pool sizes of non-structural carbohydrates

Treatments with Nod factors and TIBA also had consequences on carbohydrate metabolism in mycorrhizal roots (Fig. 2). The starch content of mycorrhizal roots was little affected by Nod factors or hormones applied singly, but when NodNGR[Ac] (0·1 μM) was applied in combination with TIBA (50 μM), in the presence or absence of kinetin (0·1 μM), it was distinctly higher (*c.* 10 mg g^{-1} d. wt) than in control roots or in roots treated with any of these effectors alone (*c.* 3 mg g^{-1} d. wt; Fig. 2*a*). In roots treated with sulphated or acetylated Nod factors alone, a considerable decrease in the pool size of sucrose, from *c.* 5 < % d. wt to < 50 %, was observed (other major soluble carbohydrates were glucose, fructose and raffinose: data not shown). In

Table 1. *Effects of TIBA on growth and nodulation of* Lablab purpureus

Treatment	Shoot f. wt (g)	Nodule f. wt (g)	Nodule number (per plant)	Nitrogenase activity (nkat g^{-1})
Control	$7{\cdot}0 \pm 0{\cdot}9$	$1{\cdot}2 \pm 0{\cdot}20$	89 ± 17	$0{\cdot}3 \pm 0{\cdot}06$
TIBA	$2{\cdot}5 \pm 0{\cdot}2$	$0{\cdot}4 \pm 0{\cdot}04$	202 ± 11	< 0·05
α	< 0·05	< 0·1	< 0·01	< 0·05

Plants were inoculated with *Rhizobium* NGR234, grown under phytotron conditions in Magenta jars with or without 50 μM TIBA, and harvested at the onset of fruiting of the control plants. The values given are means ± SE for three independent plants. The data were tested for significance using *t*-tests and the error probability α is indicated.

Table 3. *Effects of growth regulators on growth and mycorrhizal colonizaztion in* Lablab purpureus

Treatment	Shoot biomass (g f. wt)	Root biomass	Mycorrhizal colonization (% of root length)	Sporocarps (per plant)
Control	4·0±0·4 b	2·1±0·5 ab	30±5 a	10±5
NodNGR[Ac]	2·7±0·1 a	2·8±0·2 bc	59±4 b	> 100
NodNGR[S]	3·8±0·4 ab	3·0±0·3 bc	46±9 b	> 100
Kinetin	4·5±0·5 b	4·2±0·4 c	36±7 ab	> 100
NodNGR[Ac] +kinetin	2·9±0·7 ab	3·2±0·4 bc	49±5 b	> 100
TIBA	2·7±0·3 a	1·6±0·4 a	54±11 b	> 100
NodNGR[Ac] +TIBA	2·8±0·5 a	1·8±0·4 a	65±14 b	> 100
TIBA+kinetin	3·9±0·9 ab	2·8±0·2 abc	46±9 b	> 100
NodNGR[Ac] TIBA+kinetin	2·9±0·2 ab	2·2±0·05 ab	59±11 b	> 100

Plants were inoculated with *Glomus mosseae* and grown in Magenta jars. One week later, 0·1 μM Nod factors or kinetin and 50 μM TIBA were added to the nutrient solution. Data represent means±SE of three to six plants, harvested 4 wk later. Effects of the treatments on mycorrhization were checked for significance using Fisher's protected LSD test after a convenient transformation of the raw data. Data followed by the same letter are not significantly different ($\alpha = 0{\cdot}05$; ANOVA followed by Fisher's protected LSD-test).

'thick roots' elicited by the additional application of TIBA, this effect was reversed (Fig. 2*b*). The amount of fungus-borne disaccharide trehalose (undetectable in sterile roots) was close to the limit of detection in control roots (*c.* 0·06 mg g^{-1} d. wt), but increased to 1·7 mg g^{-1} d. wt following treatments with Nod factors and TIBA, alone and in combination (Fig. 2*c*). However, the trehalose content was not correlated with the degree of mycorrhizal colonization of the root (compare Table 3 with Fig. 2*c*).

Trehalase activity in mycorrhizal roots

In mycorrhizal roots without any further treatment, trehalase activity was *c.* 0·2 μkat g^{-1} protein (twice as much as in sterile roots). Upon treatment with TIBA alone, or together with kinetin, the activity was slightly increased. When TIBA was added together with acetylated Nod factor, the activity was stimulated *c.* 5-fold. This increase was significant when compared with the treatments of Nod factor, TIBA and kinetin individually, and all treatments including kinetin (Fig. 3).

DISCUSSION

The mutualistic symbioses of rhizobia and arbuscular mycorrhizal fungi with plants share many common features. (Iso)flavonoids, for example, induce the production of rhizobial Nod factors (Fellay *et al.*, 1995) and stimulate the colonization of the host root by mycorrhizal fungi (Nair, Safir & Siqueira, 1991; Xie *et al.*, 1995). Moreover, non-nodulating legume mutants are sometimes unable to be colonized by mycorrhizas (Duc *et al.*, 1989; Bradbury, Peterson & Bowley, 1991). We have shown that NodNGR[Ac], but not NodNGR[S] strongly stimulate mycorrhizal colonization when added to soybean roots (Xie *et al.*, 1995). Here we show that both Nod factors clearly stimulate mycorrhizal colonization of *L purpureus* roots. Furthermore, they dramatically enhance the formation of sporocarps. This effect can be mimicked by application of the auxin transport inhibitor, TIBA. In this case, however, the morphology of the root system is strongly altered, so that the total fungal biomass per plant does not exceed the total fungal biomass in control roots. However, in pea and soybean, TIBA stimulated relative mycorrhizal infection without these marked effects on root morphology (data not shown). Since the supply of mycorrhizal inoculum (one third of the pot volume) was clearly not limiting, we favour the hypothesis that the higher degree of mycorrhizal infection in TIBA-treated roots is not due to mere growth effects, but rather to a higher ability of the root tissue to be infected. The infection results are in good agreement with the alterations in the carbohydrate pools of the roots. It is well known that mycorrhizal roots constitute a stronger sink for assimilates than non-mycorrhizal roots (higher respiration rates and exudation; see for review Smith & Gianinazzi-Pearson (1988)). Treatments favouring mycorrhizal development should increase this sink strength. Indeed, as a consequence of the Nod factor treatments, contents of the fungal disaccharide trehalose increase and sucrose, the major non-structural plant carbohydrate, decrease (only in roots of normal morphology) thus indicating an alteration in carbon partitioning in favour of the fungus. The fungus is now able to form abundant sporocarps and accumulates compounds like trehalose. Taken together, these results clearly indicate that Nod factors and TIBA not only trigger the morphogenesis of pseudonodules and 'thick-root-tips', but also act as strong stimuli in mycorrhizal interactions, perhaps by altering source–sink relationships in carbohydrate

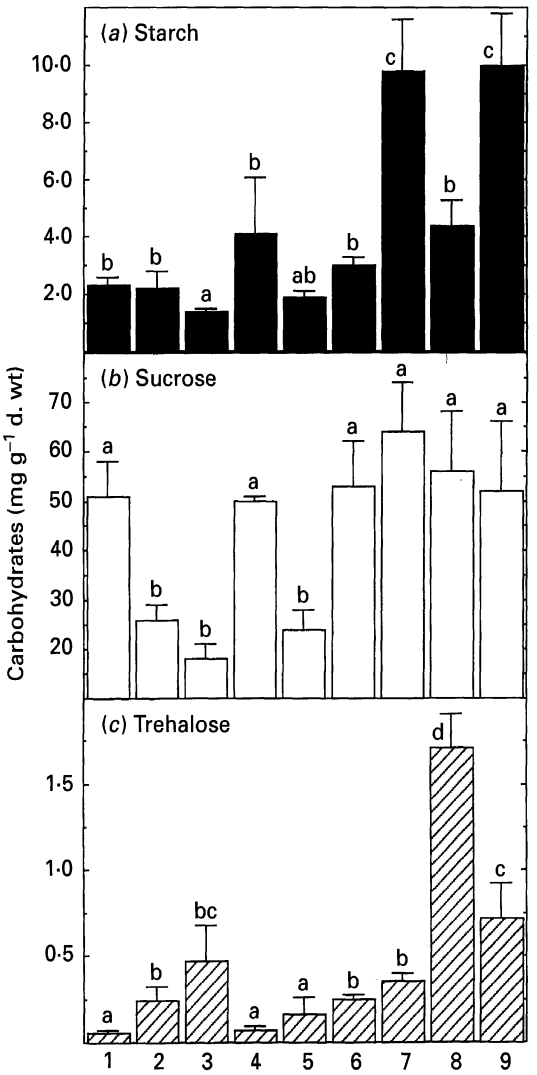

Figure 2. Starch (a), sucrose (*b*), and trehalose (*c*) contents in mycorrhizal roots of *Lablab purpureus*. Plants were inoculated with *Glomus mosseae*. One week later, the growth effectors were added to the nutrient solution at the following concentrations: 0·1 μM for Nod factors, 0·1 μM for kinetin and 50 μM for TIBA. Data represent means ± SE for three plants, harvested 4 wk later. Data followed by the same letter are not significantly different ($\alpha = 0{\cdot}05$; ANOVA followed by Fisher's protected LSD-test). 1, control; 2, NodNGR[S]; 3, NodNGR[Ac]; 4, Kin; 5, NodNGR[Ac]+Kin; 6, TIBA; 7, NodNGR[Ac]+TIBA; 8, TIBA +Kin; 9, NodNGR[Ac] +TIBA +Kin.

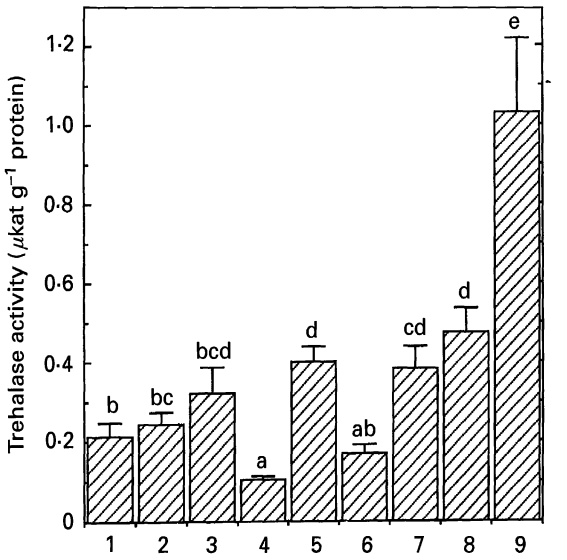

Figure 3. Effects of *tri*-iodobenzoic acid (TIBA) and Nod factors on trehalase activity in mycorrhizal *Lablab purpureus* roots. Plants were inoculated with *Glomus mosseae* and grown for 4 wk in Magenta jars (mycorrhizal inoculum:sand:loam/1:1:1) without effectors (control) or with 50 μM TIBA (TIBA), 0·1 μM NodNGR[Ac], 0·1 μM NodNGR[S] alone or in combination. Mean values and SE are given for three or six independent plants. Data followed by the same letter are not significantly different ($\alpha = 0{\cdot}05$; ANOVA followed by Fisher's protected LSD-test). 1, control; 2, NodNGR[S]; 3, NodNGR[Ac]; 4, Kin; 5, NodNGR[Ac] + Kin; 6, TIBA; 7, NodNGR[Ac] + IBA; 8, TIBA +Kin; 9, NodNGR[Ac] +TIBA +Kin.

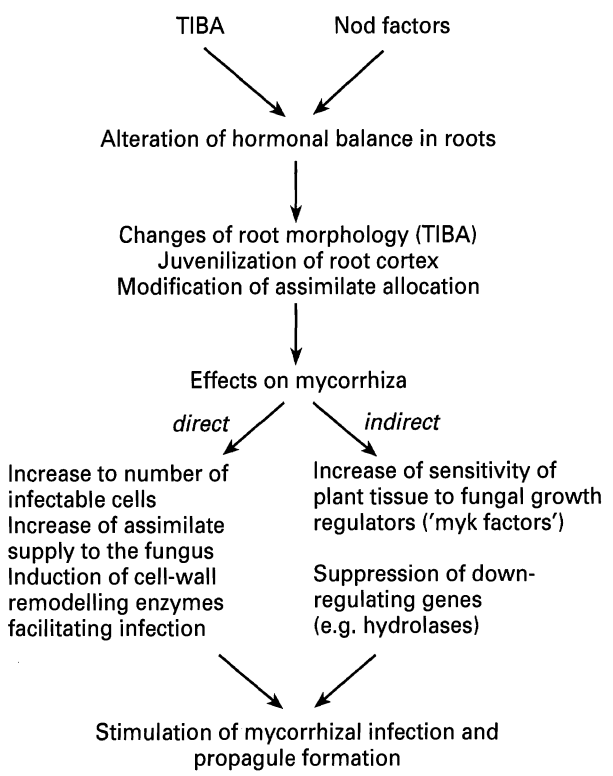

Figure 4. Scheme summarizing hypothetical effects of TIBA and Nod factors on mycorrhiza.

partitioning. The role of trehalase in this context is unclear. Interestingly, trehalase is stimulated in young, meristematic tissues (e.g. root tips, pseudonodules, pollen; Müller *et al.* (1995*a*), and in mycorrhizal roots as compared with non-mycorrhizal roots (Schellenbaum *et al.*, 1998) where > 90% of total activity is likely to be due to a plant-borne enzyme (Müller *et al.*, 1992; unpublished). Recent results suggest that this plant enzyme plays a role in degrading exogenous (or endogenous?) trehalose (or related compounds?) which could interfere with carbohydrate partitioning (unpublished).

A phytohormone-like role of Nod factors is supported by recent reports which show that Nod-factor-type molecules are also recognized by non-legumes (De Jong *et al.*, 1993; Staehelin *et al.*, 1994; Röhrig *et al.*, 1995). The mode of action could be direct, or indirect, by increasing the hormone sensitivity of specific root cells (see Fig. 4 and Hirsch & Fang (1994)). Therefore, it is possible that Nod

factors influence developmental processes ranging from mycorrhizal colonization to nodulation and cell differentiation of plants in general.

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