PHYSIOLOGIA PLANTARUM 90: 86–92. 1994
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ISSN 0031-9317

Trehalose and trehalase in root nodules from various legumes

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Müller, J., Xie, Z.-P., Staehelin, C., Mellor, R. B., Boller, T. and Wiemken, A. 1994. Trehalose and trehalase in root nodules from various legumes. – Physiol. Plant. 90: 86–92.

Nitrogen-fixing (effective) nodules from various legume-*Rhizobium* combinations were analyzed for trehalose and other soluble carbohydrates using gas chromatography and for trehalase activity using biochemical assays. Whereas the bacterial disaccharide trehalose was present only in the minority of the nodules, trehalase activity was found in all of them. Extracts from determinate nodules had a higher trehalase activity than extracts from indeterminate nodules. More detailed studies were done on soybean nodules formed in interactions with two effective and 5 ineffective *Bradyrhizobium japonicum* strains. Only in effective soybean nodules colonized by the strain 61-A-101 was trehalose a major soluble carbohydrate. Irrespective of the wildtype strains used, effective soybean nodules contained about 10 nkat trehalase g^{-1} fresh weight, whereas the ineffective nodules colonized by mutant strains derived from these wildtype strains contained 2 to 30 times less trehalase. However, a clear correlation between trehalose content and trehalase activity could not be established.

Key words – Determinate and indeterminate nodules, effective and ineffective nodules, inositol, nodule symbiosis, pinitol, trehalase, trehalose.

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Introduction

Trehalose (α-D-glucopyranosyl-[1–1]-α-D-glucopyranoside) is a common disaccharide in various organisms including bacteria, fungi, algae and articulates (Elbein 1974). It plays a role in storage of reduced carbon and, in addition, might act as an important protectant against the deleterious effects of various stresses, particularly desiccation (Crowe et al. 1984, Wiemken 1990). Trehalose is not usually present in higher plants and may be deleterious for some plant tissues (Veluthambi et al. 1981, Wagner et al. 1986), but recently it was reported that trehalose was present in *Myrothamnus flabellifolia*, a ressurrection plant in which leaves can be reversibly desiccated (Bianchi et al. 1993). In addition, trehalose occurs in plant tissues colonized by microorganisms, such as mycorrhizal roots (Schubert et al. 1992) and root nodules (Philips et al. 1984). Soybean nodules have been well studied in this regard.

In effective (nitrogen-fixing) soybean root nodules, trehalose has been described as one of the major carbohydrates (Streeter 1987). So far as is known, it is synthesized only by bacteroids (Streeter 1985). Upon nodule fractionation, trehalose has been found not only in the bacteroids but also in the soluble fraction of the plant cell (Streeter 1987) and it was recently postulated that trehalose may play a role 'as symbiotic determinant' (Mellor 1992).

Soybean nodules also contain trehalase activity (Streeter 1982, Mellor 1988). The soybean nodule trehalase has been characterized recently by Müller et al. (1992) and is probably a host-encoded, nodule-stimulated glycoprotein (see also Kinnback and Werner 1991). It was postulated that it could be induced by trehalose secreted by the bacteroids (Mellor 1992).

To determine whether there is a correlation between trehalose content and trehalase activity, we analyzed root nodules from a variety of *Rhizobium*-legume-associa-

Received 7 June, 1993; revised 13 September, 1993

Tab. 1. Nodule types (Sprent 1980, own observations), trehalose content and trehalase activity (pH 6.3; 37°C) of nodules and non-infected roots of various legume species. The nodules were colonized by the respective microsymbionts. Soybean (*G. max*) plants were infected with *B. japonicum* 61-A-101. Means ± SE is given for three independent Leonard jars. A, Amide exporting nodules; U, ureide exporting nodules; D, nodules of determinate type; I, nodules of indeterminate type. Sources of seeds and strains: [a], W. Broughton, University of Geneva (Switzerland); [b], Semences UFA (Bussigny, Switzerland); [c], Samen Mauser (Winterthur, Switzerland); [d], Samen Wyss (Basel, Switzerland); [e], D. Werner, Univ. of Marburg (Germany); [f], Ditzler AG, Möhlin (Switzerland); [g], Tourneur, Montauban (France); [h], Deutsche Sammlung für Mikroorganismen, Göttingen (Germany); [i], B. Lugtenberg, Univ. of Leiden (Netherlands); [k], D. Le Rudulier, Univ. of Nice (France).

Host plant	Symbiont strain	Nodule type	Trehalose content (mg [g DW]$^{-1}$)	Trehalase activity (nkat [g FW]$^{-1}$)	
				Nodules	Roots
Leucaena leucocephala L.[a]	*Rhizobium* sp. NGR 234[a]	A, I	<0.05	101.0±7.2	0.6±0.10
Lablab purpureus L.[a]	*Rhizobium* sp. NGR 234	U, D	0.6±0.1	50.7±0.5	0.2±0.10
Psophocarpus tetragonolobus L.[a]	*Rhizobium* sp. NGR 234	U, D	3.2±1.0	44.3±2.0	0.2±0.04
Vigna unguiculata (L.) Walp. cv. Red Caloona[a]	*Rhizobium* sp. NGR 234	U, D	<0.05	16.5±2.0	0.1±0.05
Glycine max (L.) Merr. cv. Maple Arrow[b]	*B. japonicum* (see Table 2)	U, D	<0.05	13.2±1.2	0.2±0.05
Phaseolus vulgaris L. cv. Contender[c]	*R. phaseoli* TAL 1383[h]	U (A), D	14.2±1.7	11.3±1.8	0.3±0.10
Lupinus albus L.[d]	*B. lupinii* TAL 355[h]	A, D	8.9±0.6	3.3±0.6	0.1±0.03
Cicer arietinum L.[e]	*R. ciceri* IC 2091[e]	A, I	<0.05	4.4±0.1	0.3±0.05
Pisum sativum L. cv. Dunkelgrüne Markerbse[f]	*R. leguminosarum* PRE[c]	A, I	0.9±0.2	1.6±0.3	0.2±0.04
Trifolium repens L. cv. Ladino[c]	*R. trifolii* WU 95[c]	A, I	<0.05	1.2±0.2	0.1±0.03
Vicia faba L.[d]	*R. leguminosarum* f. sp. *viciae*[i]	A, I	2.0±0.5	1.0±0.2	0.1±0.02
Medicago sativa L. cv. Du Puits[g]	*R. meliloti* 102F34[k]	A, I	<0.05	0.6±0.1	0.1±0.01

tions. In addition, we conducted a more detailed study of soybean nodules colonized by effective (fix$^+$) and ineffective (fix$^-$) *Bradyrhizobium* strains. We analyzed the soluble carbohydrates and measured the trehalase activity in all nodules.

Materials and methods

Plants and bacteria

The spectrum of legumes tested included plants from tropical and temperate regions forming determinate or indeterminate nodules with their respective symbionts (Tab. 1). In addition, soybean plants (*Glycine max* L. Merr. cv. Maple Arrow) infected with effective and ineffective *Bradyrhizobium japonicum* strains were tested (Tab. 2).

Establishment of symbioses and harvest of nodules

The seeds of all plants were surface-sterilized with H_2O_2 (30%, w/v), germinated on agar plates, infected with a stationary-phase culture of their respective symbiont and put into Leonard jars (Staehelin et al. 1992). The soybean plants were grown as described by Staehelin et al. (1992). The other plants were grown under greenhouse conditions at about 22°C and a 14-h photoperiod. To obtain uninfected roots, sterile seedlings were planted into Leonard Jars containing a nutrient solution supplemented with 5 m*M* KNO_3. The *Rhizobium* and *Bradyrhizobium* strains were grown to stationary phase in 20E-medium at 27°C on a rotary shaker at 140 rpm (Staehelin et al. 1992). The nodules were harvested as indicated (time curve), at the onset of fruit formation (5–6 weeks after planting), or after 3 months in the case of the tree *Leucaena*, frozen immediately, and stored at –20°C.

Tab. 2. Characteristics of the *Bradyrhizobium japonicum* strains used to infect soybean (*Glycine max* [L.] Merr. cv. Maple Arrow). Sources: [a], D. Werner, Univ. of Marburg (Germany); [b], H. Hennecke, ETH Zurich (Switzerland).

Strain	Characteristics	Reference
61-A-101[a]	fix$^+$-wildtype	Stripf and Werner 1978
RH 31 Marburg[a]	fix$^-$-mutant of A 101	Werner et al. 1984
61-A-24[a]	fix$^-$-wildtype	Werner et al. 1980
USDA 110 *spc4* (110)[b]	fix$^+$-wildtype	Regensburger and Hennecke 1983
A3[b]	fix$^-$-mutant of 110 (Δ *nif D*)	Hahn et al. 1984
A9[b]	fix$^-$-mutant of 110 (Δ *nif A*)	Fischer et al. 1986
Δ *El–8dl*[b]	fix$^-$-mutant of 110 (Δ *cluster I*)	Hahn and Hennecke 1987

Tab. 3. Trehalose content of free-living *Rhizobium* and *Bradyrhizobium* strains grown to stationary phase in liquid culture. Means ± SE are given for three independent extractions.

Strain	Trehalose content, mg (g DW)$^{-1}$
Rhizobium sp. NGR 234	0.1 ± 0.03
Rhizobium phaseoli TAL 1383	< 0.05
Rhizobium leguminosarum PRE	< 0.05
Rhizobium leguminosarum f. sp. *viciae*	0.6 ± 0.05
Rhizobium ciceri IC 2091	< 0.05
Rhizobium trifolii WU 95	< 0.05
Rhizobium meliloti 102F34	< 0.05
Bradyrhizobium lupinii TAL	7.4 ± 0.3
Bradyrhizobium japonicum:	
61-A-101	7.0 ± 1.2
RH 31 Marburg	8.1 ± 0.5
61-A-24	8.0 ± 0.6
110 *spc 4* wt	11.3 ± 0.9
A3	11.8 ± 0.4
A9	21.2 ± 0.8
Δ *El–8dl*	9.0 ± 1.4

Determination of carbohydrates

For the assay of carbohydrates (Schubert et al. 1992), nodules were lyophilized, weighed, and ground in methanol (80% v/v; 50 ml g^{-1} dry weight) containing insoluble polyvinylpyrrolidone (SERVA, Heidelberg, Germany) and 50 μg mannoheptulose (internal standard). The soluble carbohydrates were extracted at 60°C for 10 min followed by centrifugation (13 000 *g*, 10 min). The extraction was repeated three times. The supernatants were collected and vacuum-dried. To analyze the carbohydrate content of bacteria, the strains were grown to stationary phase, harvested by centrifugation (10 000 *g*, 10 min) and washed twice in ice-cold sterilized tap-water. The pellets were lyophilized, weighed and extracted (as for the plant material).

The residues extracted from 10–20 mg dry weight of nodules or bacteria were resuspended in 0.6 ml double distilled water. To remove charged compounds, 50 μl of wet mixed-bed ion-exchanger (Serdolit micro blue and red 2:1 [v/v], SERVA, Heidelberg, Germany) were added. The tubes were vortexed and centrifuged (13 000 *g*, 10 min). The supernatants were lyophilized, redissolved in methanol (50%, v/v) and transferred to gas chromatography (GC) vials. The solvents were removed by vacuum-drying. Water was completely removed by twice adding pure methanol followed by drying at 80°C.

The vials were closed tightly and 50 μl pyridine containing 625 μg hydroxylamine and 50 μg phenyl-β-glucopyranoside (derivatization standard) were added to each sample. The vials were incubated for 30 min at 80°C. For the derivatization, 50 μl N-methyl-N-trimethylsilyl-heptafluorobutyramide containing 1% (v/v) trimethyl-chlorsilane were added. The vials were incubated for 30 min at 80°C.

Gas chromatography of the derivatized carbohydrates was performed on a Shimadzu 14A gas chromatograph with a chromosorb W-HP (silicate) column coated with OV-17 (5%). The injections (4 μl) were done using an auto-injector AOC 14 with an autosampler AOC 1400. The carbohydrate derivates were eluted from the column using temperatures rising from 70°C to 300°C. One run was performed in 40 min. The carbohydrates were identified by comparison with known standards and quantified relative to the internal standard mannoheptulose. The detection limit was lower than 50 μg carbohydrate g^{-1} dry weight.

Determination of trehalase activity

To extract proteins for the determination of trehalase activity, frozen nodule samples were ground in MES (K^+) buffer (0.1 *M*, pH 6.3) containing ethylene-diamine tetraacetic acid and phenyl-methyl-sulfonylfluoride (2 m*M* each). The buffer-sample ratio was 2 ml g^{-1} fresh weight. Trehalase was assayed by incubating enzyme aliquots in MES (K^+) buffer (pH 6.3, 50 m*M*) containing 10 m*M* trehalose at 37°C for 15 to 30 min. The assay was stopped by boiling and the glucose generated was determined with the glucose oxidase-peroxidase method using a test kit (Boehringer, Mannheim, Germany). Substrate and enzyme blanks were subtracted (Müller et al. 1992). In all nodule extracts, after centrifugation of the extracts at 48 000 *g* for 20 min, trehalase activity was found exclusively in the supernatant. The trehalase activity was not altered by freezing the nodules or nodule extracts.

Results

Trehalose content of rhizobia and bradyrhizobia in liquid cultures

All *Rhizobium* and *Bradyrhizobium* strains used in this work were grown to stationary phase. Carbohydrates were extracted as described and analyzed by gas chromatography. Trehalose was the only major compound detected. All *Bradyrhizobium* strains contained about 10 mg trehalose g^{-1} dry weight when grown to stationary phase. The strain A9 (Δ *nifA*) contained twice as much trehalose as the corresponding wildtype, 110 *spc 4*. The *Rhizobium* strains contained little or no detectable trehalose (Tab. 3).

Trehalose and trehalase activity in root nodules of various legumes

In the combinations tested, trehalose was a major carbohydrate (≈ 1% dry weight) only in soybean nodules colonized by *B. japonicum* 61-A-101 and in French bean nodules colonized by *R. phaseoli* TAL 1383 (Tab. 1). It was also present, at levels between ca 0.1 and 0.3% dry weight, in nodules of *Lablab, Trifolium* and *Vicia* colonized by their respective symbionts. All other nodules contained only little or no detectable trehalose. However, trehalase activity was found in all nodules. In all cases,

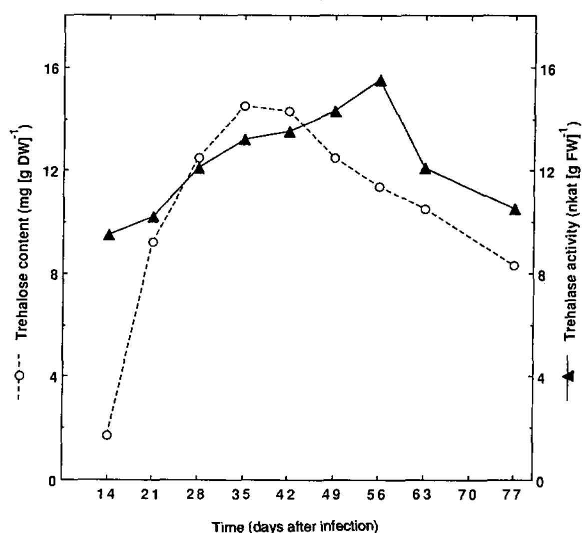

Fig. 1. Trehalose content and trehalase activity (pH 6.3; 37°C) in soybean nodules colonized by the effective *Bradyrhizobium japonicum* strain 61-A-101 during the nodule development.

trehalase activity was nodule-stimulated, i.e. much higher than in uninfected roots (Tab. 1). Very high activities were found in nodules of *Leucaena leucocephala, Lablab purpureus* and *Psophocarpus tetragonolobus*. These nodules could thus be sources for the enzyme. No correlation between trehalase activity and trehalose content of the nodules was detected. Trehalase activity in nodules of the herbaceous plants was significantly higher ($\alpha = 0.05$) in determinate (tribe Phaseolae) than in indeterminate nodules (23 ± 17.9 vs 1.8 ± 1.35 nkat g^{-1} fresh weight).

Trehalose and other carbohydrates in effective and ineffective soybean nodules

In mature soybean root nodules colonized by the effective strain *Bradyrhizobium japonicum* 61-A-101, trehalose was a major carbohydrate (14–15 mg g^{-1} dry weight) about 5 weeks after infection (Fig. 1). Nodules of the same age colonized by the ineffective mutant RH 31 Marburg derived from 61-A-101 and by the ineffective wildtype 61-A-24, contained 2 and 5 times less trehalose, respectively (Fig. 2A), a feature also of nodules colonized by the ineffective strain Δ *E1–8d1*. These nodules also contained only 20% as much trehalose as those raised with their isogenic effective wildtype 110 *spc4*. Two other ineffective mutants of this strain, A3 (Δ *nifD*) and A9 (Δ *nifA*), did not depress the trehalose content of the nodules which they colonized. Nodules formed with strain A9 contained twice as much trehalose as nodules formed with the corresponding wildtype bacteria (Fig. 2A).

The mean sucrose content of ineffective nodules in all cases was higher than in effective nodules colonized by *B. japonicum* 61-A-101 or 110 *spc4*. The differences were statistically significant ($\alpha = 0.05$) except for Δ *E1–8d1*. The ineffective nodules colonized by the strains A3 and A9 contained 2 and 3 times more sucrose than effective nodules, respectively (Fig. 2B). The nodules con-

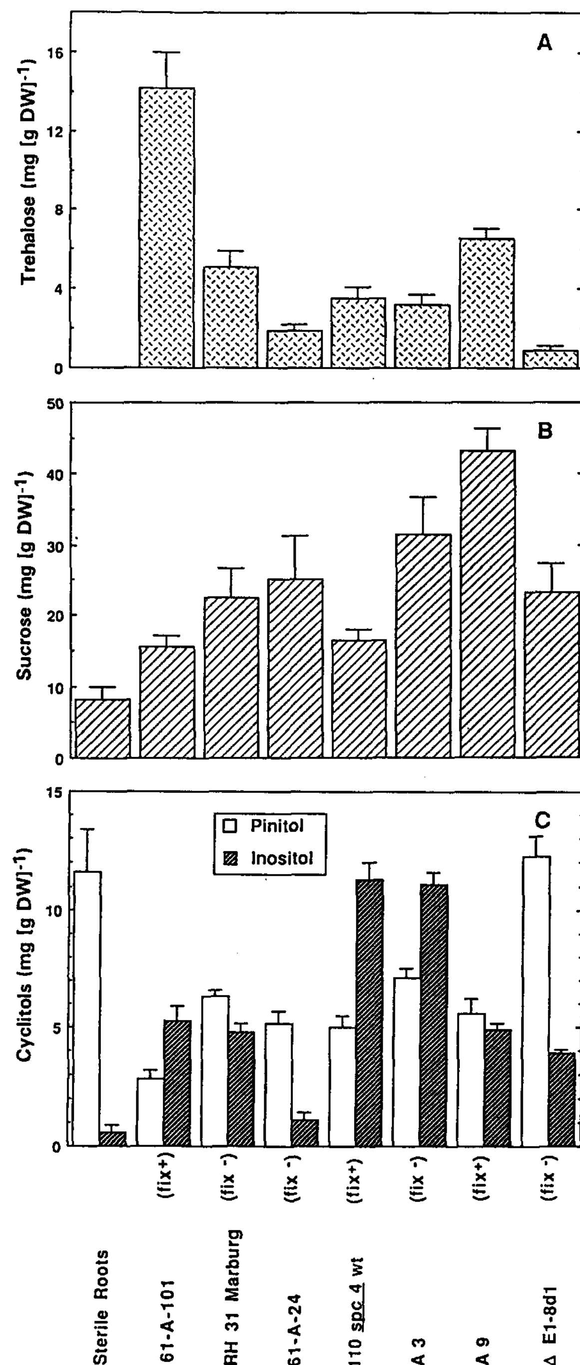

Fig. 2. Soluble carbohydrates in soybean nodules colonized by effective (61-A-101 and 110 *spc4* wildtype) and ineffective (*RH 31 Marburg*, 61-A-24, A3, A9, Δ *E1–8d1*) *B. japonicum* strains compared with sterile roots. Means ± SE are given for 3 independent jars. A, trehalose; B, sucrose; C, cyclitols. Note different scales on the abscissae.

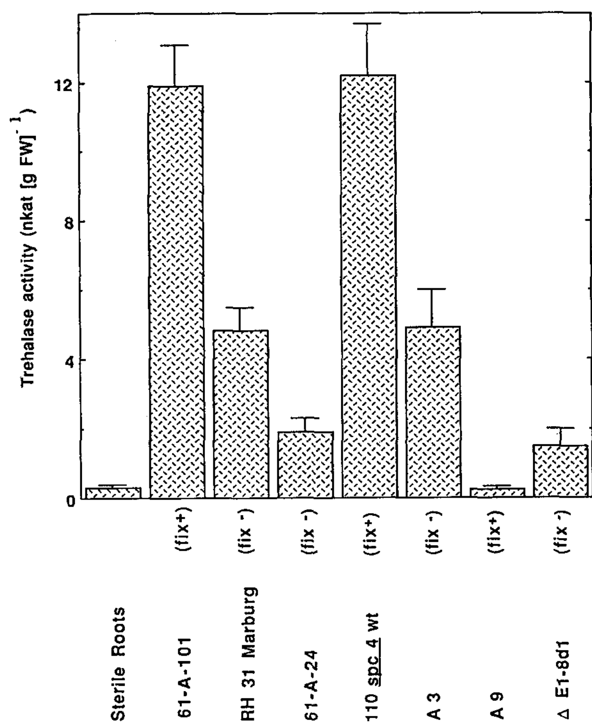

Fig. 3. Trehalase activity (pH 6.3; 37°C) in soybean nodules colonized by effective (61-A-101 and *110 spc4* wildtype) and ineffective (RH 31 Marburg, 61-A-24, A3, A9, Δ *E1–8d1*) *B. japonicum* strains compared with sterile roots. Means ± SE are given for 3 independent jars.

tained about twice as much sucrose g^{-1} dry weight as the uninfected roots. Pinitol, the major soluble carbohydrate in sterile roots, was lower in the effective nodules, whereas a related compound, the non-methylated inositol was present at higher levels (Fig. 2C). The ineffective as compared with the effective nodules always contained more pinitol and less inositol.

Trehalase activity of effective and ineffective soybean nodules

In the effective soybean nodules formed with two different wildtype strains, trehalase activity reached about 12 nkat g^{-1} fresh weight which is about 30 times more than in the uninfected roots (Fig. 3). A similar activity was found in nodules from soybean grown in the fields at two different locations near Basel, Switzerland (data not shown). Trehalase activity changed only little during the development of nodules (Fig. 1). All ineffective nodules contained conspicuously less trehalase activity than effective ones (Fig. 3). In particular, the nodules colonized by the ineffective strains 61-A-24 and Δ *E1–8d1* contained only 10% of the activity present in effective nodules and the A9-nodules contained only as much trehalase as uninfected roots.

Discussion

Bradyrhizobium strains accumulated large amounts of trehalose in stationary cultures (about 1% of dry weight), whereas stationary *Rhizobium* cells contained only very little. Trehalose is presumed to act as a stress protectant (Crowe et al. 1984, Wiemken 1990). Hence the high trehalose accumulation by *Bradyrhizobium* strains, as compared with the *Rhizobium* strains, may be related to the higher survival upon prolonged storage in water of *Bradyhizobium* than of *Rhizobium* strains (Streeter 1985). This does not mean that *Rhizobium* strains are unable to synthesize trehalose. The strain *R. meliloti* SU-47 accumulated trehalose when exposed to high osmolarity (Breedveld et al. 1990) and several other *Rhizobium* strains are known to accumulate trehalose when grown under low oxygen partial pressure (Hoelzle and Streeter 1990).

In the legume nodules tested in this study, trehalose was a major carbohydrate of nodules only in the combinations *Glycine max – B. japonicum* 61-A-101 and *Phaseolus vulgaris – R. phaseoli* TAL 1383. Its content was much lower in other nodules and even below the detection limit (<0.05 mg g^{-1} dry weight) in some of the effective nodules. Therefore, it seems unlikely that trehalose plays an essential role in the nitrogen fixing symbiosis (Mellor 1992). Moreover, the low oxygen partial pressure of nodules (Minchin et al. 1985) which induces accumulation of trehalose in free-living rhizobia (Hoelzle and Streeter 1990) does not appear to do so in the nodules.

In soybean, ineffective nodules colonized by some mutant strains contained less trehalose than effective nodules colonized by their respective isogenic wildtype (RH 31 and 110 *spc4* Δ *E1–8d1*). This appears not to be due to a reduced allocation of assimilates from the shoots to the nodules, since the ineffective nodules in general contained more sucrose and pinitol than effective nodules. As non-nitrogen fixing bacteroids are expected to consume less energy than those that fix N_2, transformation of sucrose to organic acids might be inhibited by negative feedback, causing an increase in the sucrose pool size.

Based on earlier results (Streeter 1987), it was postulated that nodule trehalase could be induced by trehalose released from the bacteroids (Mellor 1992), but we found no correlation between trehalose content and trehalase activity. A nodule-stimulated trehalase activity has been found in alfalfa nodules colonized by *R. meliloti* 102F34, a strain which did not accumulate trehalose even under high salt conditions (Fougère et al. 1991).

Effective soybean nodules contained a higher trehalase activity than ineffective nodules. Nodules colonized by the ineffective wildtype strain *B. japonicum* 61-A-24 are greenish and contain no active leghemoglobin (Werner et al. 1980). A hypersensitive reaction is induced in these nodules (Staehelin et al. 1992) and this study shows that they contain only little trehalase activity. A similar obser-

vation was made with greenish nodules colonized by *B. japonicum* 110 *spc4* Δ *El–8dl* and 110 *spc4* A9 (Δ *nif A*). Thus, trehalase might be confined to nodule tissue actually involved in nitrogen fixation. This would agree with the observation that determinate nodules contain more trehalase than indeterminate nodules (except *Leucaena*), since in indeterminate nodules only a part of the tissue contains leghemoglobin and is fixing nitrogen (Sprent 1980, Hirsch 1992).

What is the function of trehalase in plants? It was described for the fist time in pollen of *Lilium longiflorum* (Gussin et al. 1969). We found that trehalase activities from this source and from pollinia of the orchid *Cymbidium* sp. were inhibited by the trehalase inhibitor validamycin (Asano et al. 1987) at low concentrations (unpublished data). Trehalase activity has also been described in tissue and cell cultures of various gymnosperms, dicots and monocots (Veluthambi et al. 1981, Kendall 1990). Müller et al. (1992) found validamycin-inhibited trehalase activity in soybean callus cultures and in cell suspension cultures in which more than 90% of the total activity was in the medium (unpublished data). Since the putative substrate of this enzyme, trehalose, has not been detected in the plant tissues mentioned above, the function of trehalase is not at all clear. Its role may be that of cleaving trehalose derived from symbionts (Veluthambi 1981, see for review Mellor 1992). Alternatively, trehalose may not be the in vivo substrate of this enzyme. However, this is unlikely considering the high substrate specificity generally attributed to trehalases (Elbein 1974). We have previously partially purified and characterized validamycin-inhibited trehalase from soybean nodules. The enzyme was specifically bound to concanavalin A and thus was considered to be a plant borne glycoprotein (Müller et al. 1992).

Acknowledgements – We thank W. Broughton (Geneva), H. Hennecke and H.-M. Fischer (ETH Zurich), D. Le Rudulier (Nizza), B. Lutgenberg (Leyden) and D. Werner (Marburg) for generously providing legume seeds and *Rhizobium* strains. We also thank K. Ineichen for valuable help with the gas chromatograph. This work was supported by the Swiss National Foundation grants 31-27923.89 (to A. W.) and 30-21730.88 (to R. M. and T. B.).

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Edited by C. H. Bornman