

The Effect of Exogenous Carbohydrates on Nitrogen Fixation and *hetR* Expression in *Nostoc* PCC 9229 Forming Symbiosis with *Gunnera*

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Abstract

The cyanobacterium *Nostoc* PCC 9229 forms an intracellular nitrogen-fixing symbiosis with the angiosperm *Gunnera*. In symbiosis the cyanobacterium is enclosed in darkness and receives carbon from the plant in an unknown form. Out of five putative plant carbohydrate sources tested *in vitro*, fructose and glucose were found to support nitrogen fixation in darkness. The other three dextrin, sucrose and *Gunnera* sp. mucilage could not induce nitrogenase activity in darkness. The stimulatory effect by fructose was also observed in illuminated samples. After four weeks incubation in darkness, nitrogenase was still active in cultures when fructose was added and multiple thick-walled nitrogen-fixing cells (heterocysts) were observed, and chlorophyll levels unchanged. The expression as shown by Northern blot analysis revealed that fructose influenced the gene expression of *hetR*, a gene necessary for heterocyst formation, in darkness. Fructose and glucose may therefore be the carbohydrates supplied by the host plant to induce heterocyst differentiation and nitrogen fixation in the cyanobiont *Nostoc* PCC 9229.

Keywords: Carbohydrates, *hetR*, N₂-fixation, *Nostoc* PCC 9229, symbiosis

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1. Introduction

Cyanobacteria are oxygenic, photosynthesizing prokaryotes with a world-wide distribution and according to the "endosymbiont theory", they are the progenitors of plant plastids (Margulis, 1970; Douglas, 1994). Apart from carbon fixation some species are capable of fixing atmospheric nitrogen. *Nostoc* is one such cyanobacterium, which forms specialized cells, termed heterocysts, at regular intervals along the filament where the nitrogen fixation takes place. Heterocysts have thickened cell walls which are less permeable to gases and lack the oxygen-evolving photosystem (PS) II (Wolk et al., 1994). The transcription of the heterocyst regulatory gene *hetR* increases during heterocyst differentiation (Buikema and Haselkorn, 1991), and the expression of this gene is possibly a good marker for heterocyst differentiation. Heterocyst-forming cyanobacteria form symbiosis with a wide range of organisms, such as protists, lichenized fungi and in the plant kingdom with liverworts/hornworts, a waterfern (*Azolla*), gymnosperms (Cycads) and with the angiosperm *Gunnera* (Bergman et al., 1996). All *Gunnera* species examined form symbiosis with *Nostoc*, and are therefore not dependent on external combined nitrogen for growth (Silvester and Smith, 1969).

Nostoc infects the *Gunnera* plant through stem glands (Silvester and McNamara, 1976; Johansson and Bergman, 1994) which produce an acidic, carbohydrate-rich mucilage that induces the differentiation of motile filaments without heterocyst (hormogonia) in symbiotically competent *Nostoc* strains (Rasmussen et al., 1994). When reaching the gland base, *Nostoc* is engulfed and becomes intracellular, and as a consequence a symbiotic stem tissue is formed (Silvester and McNamara, 1976). At this stage a higher number of heterocysts are formed, compared to that in free-living *Nostoc*, and these contain the nitrogen-fixing enzyme nitrogenase (Söderbäck et al., 1990). The ability for heterotrophic growth and dark nitrogen fixation is a trait present in many free-living *Nostoc* strains (Huang and Chow, 1988). Symbiotic *Nostoc* is presumed to use host-supplied carbohydrates as sole energy source, since it is known that an unknown form of carbohydrate is translocated by *Gunnera* to *Nostoc*-containing tissues (Söderbäck and Bergman, 1993). The effects of exogenous carbohydrates on nitrogen fixation in darkness in *Nostoc* strains documented to be symbiotically competent are sparse. However, fructose and glucose stimulates nitrogen fixation in darkness if added to excised symbiotic *Gunnera* tissues (Silvester and McNamara, 1976; Man and Silvester, 1994), but the influence on *hetR* expression by these compounds has not been examined.

The purpose of the present study was therefore, firstly, to search for carbohydrates functioning as promoters of nitrogen fixation activity and altered heterocyst patterns in the symbiotically competent *Nostoc* PCC 9229.

Secondly, to investigate the physiological effects of these compounds by examining the expression of *hetR*.

2. Materials and Methods

Growth conditions

Nostoc PCC 9229 isolated from *Gunnera monoica*, from New Zealand (by Dr. E. Söderbäck, Stockholm University), was used for all experiments. The *in vitro* symbiotically competent *Nostoc* PCC 9229 strain readily infects *Gunnera* under laboratory conditions (Johansson and Bergman, 1994), and has been selected as the type strain in this laboratory. Axenic stock cultures were grown on nitrate-free BG-11₀ medium (Rippka et al., 1979) and were maintained under axenic conditions with continuous cool fluorescent light of 25 to 30 $\mu\text{mol m}^{-2}\text{s}^{-1}$. Large batch cultures (1.5 l) were grown on the same medium and were stirred using a magnetic bar and flushed with moist air. The stock cultures were harvested after 3 weeks, the cells were left to sediment over night after which the supernatant was decanted and thus concentrating the cell suspension to approximately 150 ml. The culture was homogenized to single filaments in 50 ml Falcon tubes with glass beads using vortex. One or two ml of the homogenate was portioned into 100 ml bottles containing 20 ml BG-11₀ medium, supplemented with various carbohydrates. To obtain dark culture conditions, the bottles were covered with aluminum foil. Nitrogen fixation activity was measured by acetylene reduction.

Carbon sources

The mono-saccharides fructose and glucose, the disaccharide sucrose and the oligosaccharide dextrin-10 were used in the short term experiments. The dextrin-10 was derived from maize starch (maltodextrin), EC No 2329404, and will be referred to as dextrin throughout the text. In plants, the apoplastic concentrations of sucrose and hexoses are a few millimolar (Pollock and Farrar, 1996). A sugar concentration between 1 and 2 mM was used here on the free-living *Nostoc* PCC 9229 culture. For the long-term experiment the concentration of fructose was increased to 10 mM and to 5 mM of sucrose. The dextrin which is not a sugar in a transportable form in plants was used in a concentration of 1 mM in all experiments. The different molarities of sugars used in the long-term experiment were due to the different numbers of hexoses in the sugar molecule. The carbohydrate-rich *Gunnera* mucilage secreted by the stem glands (kindly provided by Dr. W. Silvester, University of Waikato, Hamilton, New Zealand) was stored at 4°C until use, the mucilage was used in concentrations

between 1 and 50% (v/v). As earlier attempts to culture *Nostoc* PCC 9229 in the dark with different concentrations of the non-axenic mucilage for more than a few days have failed (J. Wouters unpublished observations) this was avoided.

Nitrogenase activity

The *Nostoc* PCC 9229 cultures were incubated with 10% (v/v) acetylene for 1 hour before the first sampling of ethylene production was made, the second sample was taken after an additional hour of incubation. The measurements were performed as described earlier (Janson et al., 1998).

Chlorophyll measurements

Nostoc PCC 9229 cells were collected by centrifugation, the pellets ground in liquid nitrogen and the homogenate dissolved in 90% methanol. After 2 h incubation in darkness, cell debris were removed by centrifugation and absorbance of the supernatant measured with 90% methanol as blank. The chlorophyll *a* (Chl_a) content was calculated as $C(\mu\text{g/ml}) = \text{OD}_{665\text{nm}} \times 13.9$ (Tandeau de Marsac and Homard, 1988).

Northern blot

For *hetR* expression, duplicate *Nostoc* PCC 9229 cultures were used for RNA extraction. The cells were collected by centrifugation and pellets ground in liquid nitrogen. RNA was extracted with QIAGEN Midi columns (QIAGEN GmbH, Hilden, Germany), using the protocol for plant RNA. The RNA concentration was determined by the absorbance at 260 nm. Different sized RNA was separated on a formamide gel and blotted onto a Zeta-Probe nylon filter (BIO-RAD, Hercules, California, USA). Hybridization and washing was performed according to the formamide protocol described by the manufacturer. The *hetR* gene from *Nostoc* PCC 9229 (GenBank accession number X92989) was isolated earlier (A. Matveyev, F. Lotti and B. Bergman, unpublished). The *hetR* gene probe was labeled with ³²P-dCTP using the DECAprime II Kit (Ambion INC, Austin, Texas, USA). Filters were exposed onto Kodak MS film.

3. Results

Dark nitrogenase activity

After 24 h incubation in darkness the nitrogenase activity was detected in cultures supplemented with 2 mM of the mono-saccharides glucose and fructose

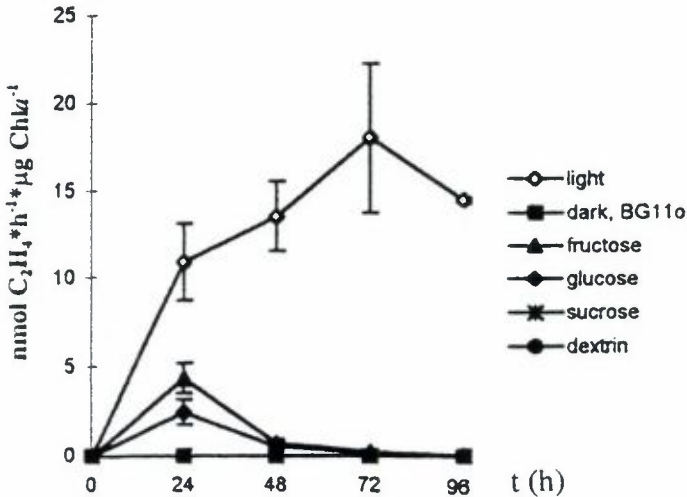


Figure 1. The response of different carbohydrates on nitrogenase activity in *Nostoc* PCC 9229 sustained in darkness. Carbohydrates were added at time zero. Note that the cultures incubated in the light showed a higher nitrogenase activity and the control cultures incubated in the dark without carbohydrates added showed no detectable activity. Bars show standard deviation from three samples.

(Fig. 1). Then the activity dropped over a period of 72 h and was no longer detectable after 96 h. The di-saccharide sucrose and the oligo-saccharide dextrin did not sustain nitrogenase activity in darkness.

The effect of light on cultures with dark nitrogenase activity

After 48 h of incubation in the dark, the addition of fructose and glucose, resulted in nitrogenase activity in darkness (Table 1), while the sucrose and dextrin did not induce nitrogenase activity. Fructose addition resulted in a greater nitrogenase activity than glucose, in both dark and light exposure. Fructose was therefore chosen to represent a mono-saccharide. The *Gunnera* mucilage could not support nitrogen-fixation in darkness or maintain the cultures in such a condition that there was a response to light, and was excluded from further experiments.

Short term responses to the addition of fructose

In order to estimate the time needed by fructose to trigger nitrogenase

Table 1. Light stimulated nitrogenase activity in dark incubated cultures. Cultures were incubated for 48 h in darkness in culture media supplemented with *Gunnnera* mucilage or carbohydrates and exposed to light ($25 \mu\text{mol m}^{-2}\text{s}^{-1}$). The values are the mean of duplicate samples with the variation in brackets of the detectable nitrogenase activity.

Carbohydrate	Nitrogenase activity ($\text{nmol C}_2\text{H}_4 \cdot \text{h}^{-1} \cdot \mu\text{g Chl}a^{-1}$)	
	Darkness	1 h light
Mucilage 1%	0	0
Mucilage 10%	0	0
Mucilage 50%	0	0
Glucose	0.5 (± 0.01)	2.3 (± 0.2)
Fructose	1.3 (± 0.4)	3.1 (± 0.6)
Sucrose	0	0
Dextrin	0	0
No sugar added	0	0

Table 2. Photoautotrophic and photoheterotrophic nitrogenase activity by the *Nostoc* PCC 9229. Cultures were sampled for nitrogenase activity after 24 h pretreatment in darkness followed by 24 h in light ($25 \mu\text{mol m}^{-2}\text{s}^{-1}$), with and without fructose. The values between brackets are the standard deviation of the mean of five samples.

Carbohydrate	Nitrogenase activity ($\text{nmol C}_2\text{H}_4 \cdot \text{h}^{-1} \cdot \mu\text{g Chl}a^{-1}$)
Fructose	6.0 (± 2.0)
No sugar added	3.1 (± 0.7)

activity in the darkness, cultures were incubated in darkness for 20 h. After adding fructose samples were withdrawn at 15 min intervals. Nitrogenase activity was first detected at 90 min after the addition and the activity thereafter increased steadily over a period of 3 h (data not shown).

To evaluate whether the effect of fructose on nitrogenase activity was restricted to culture conditions in darkness, photoheterotrophic conditions were also tested. As shown in Table 2 the addition of fructose and light to dark-incubated cultures resulted in a significant increase of acetylene reduction, compared to light only.

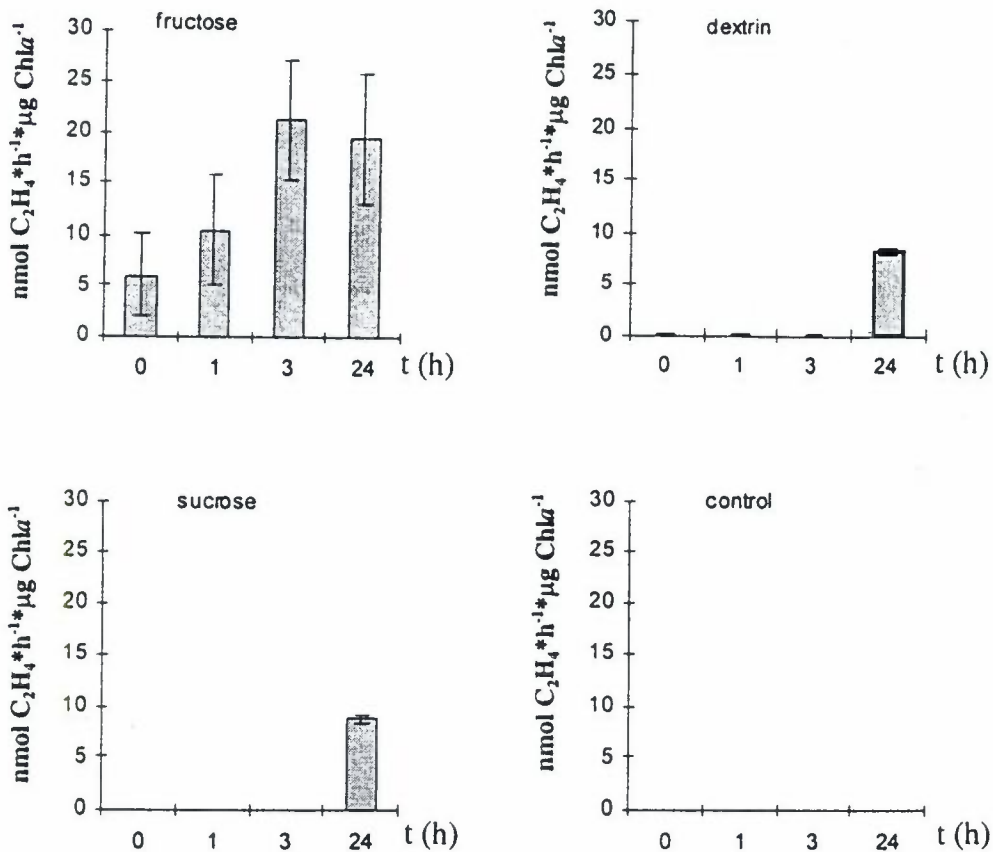


Figure 2. Long term effect of dark incubation on nitrogenase activity. Cultures of *Nostoc* PCC 9229 were kept in darkness for four weeks. The cultures were thereafter exposed to light (time zero) and samples taken at times indicated. Fructose showed activity before the onset of light, sucrose and dextrin after 24 h light exposure, the control with no addition showed no activity. Bars show standard deviation of triplicate samples.

The response to long-term incubations with carbohydrates

To investigate the physiological state of *Nostoc* PCC 9229 incubated for a longer period in darkness with an exogenous carbon source, nitrogenase activity was measured after 4 weeks incubation in darkness with exogenous carbon as only carbon source. Only fructose gave nitrogenase activity in darkness before the light incubation (Fig. 2). Light further stimulated the activity in the fructose supplemented samples, and after 24 h incubation sucrose and dextrin

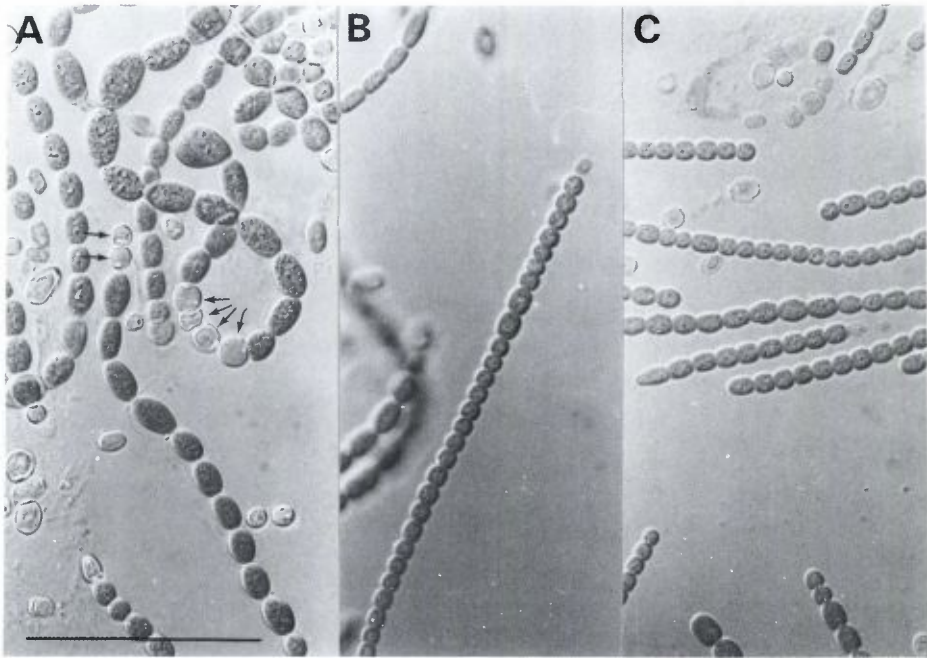


Figure 3. Morphology of filaments incubated in the dark for four weeks. Interference microscopy showing filaments from cultures treated with carbohydrates as in Fig. 2. Culture incubated in darkness with fructose (A), note the formation of multiple heterocysts (arrows) and enlarged vegetative cells. Cultures incubated in darkness with sucrose (B) and dextrin (C), both have smaller more isodiametric shaped cells, and filaments without heterocysts. Scale bar represents 50 μm .

supplemented cultures showed nitrogenase activity. Cultures that had not been supplemented with carbohydrates were not reactivated by light.

Growth in darkness

To investigate the growth of cultures incubated in darkness, the chlorophyll *a* (Chl*a*) content of the cultures, with equal amounts of inoculant, was determined. After two days in the dark the average content of the free-living *Nostoc* PCC 9229 cultures were 1.68 (\pm 52) mg Chl*a* l^{-1} . After 30 days incubation in the dark the Chl*a* content of the non-supplemented control cultures had decreased to 0.84 (\pm 0.2) mg Chl*a* l^{-1} . The cultures supplemented with fructose had 1.8 (\pm 0.92) mg Chl*a* l^{-1} , sucrose 1.72 (\pm 0.92) mg Chl*a* l^{-1}

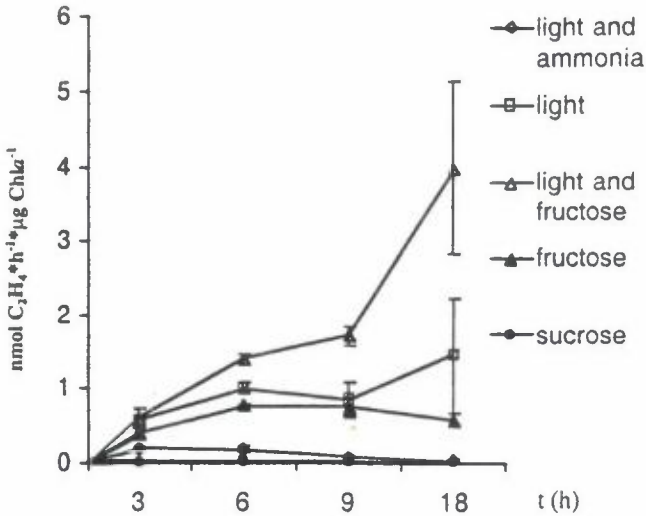


Figure 4. Nitrogenase activity by *Nostoc* PCC 9229 pre-incubated in darkness. After 20 h exposure to darkness the cultures were supplemented with light, fructose, NH₄ or sucrose at time zero. Cultures supplemented with light and NH₄ showed a low activity at 3 h, that declined to undetectable at the end of the experiment. Cultures supplemented with sucrose, but kept in darkness, showed no activity. Addition of only fructose had a similar effect as those only treated with light. Light and fructose in combination showed the highest stimulatory effect. Cultures that were not supplemented are not shown in the diagram, as shown for sucrose, these showed no activity. Bars show the standard deviation between two samples.

and dextrin 1.92 (+/- 0.92) mg Chla l⁻¹. This indicates that the chemoheterotrophic conditions used did not result in any substantial increase in Chla in *Nostoc* PCC 9229 which also is the case for cyanobacteria under symbiotic conditions (Bergman et al., 1992).

Heterocyst differentiation

The effects of carbohydrates on morphology and heterocyst differentiation in *Nostoc* PCC 9229 was also examined. After 4 weeks incubation in the dark, filaments with the typical pattern of single heterocysts separated by 10 to 20 vegetative cells were common in cultures supplemented with fructose, but

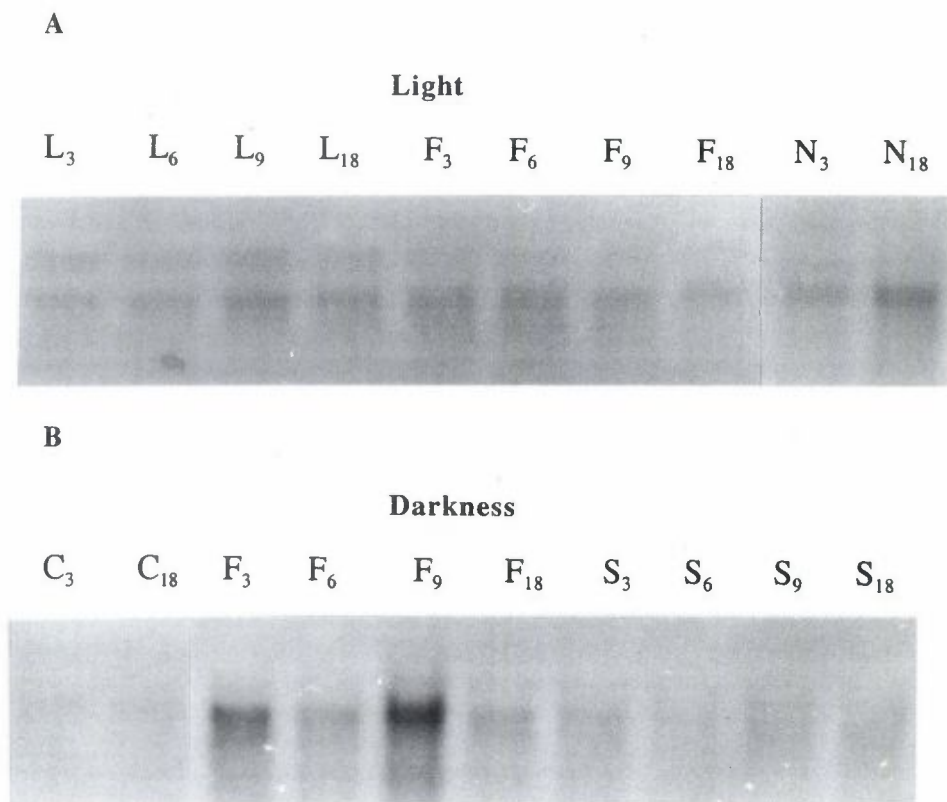


Figure 5. Expression of *hetR* in *Nostoc* PCC 9229 pre-incubated in darkness. The autoradiographs of blots containing RNA isolated from the cells used in Fig. 4 and hybridized with a gene probe for the *hetR* gene. Numbers refers to the time after the onset of the experiment when RNA was harvested. Cultures supplemented with only light, lanes marked L, and cultures that in addition where supplemented with fructose or NH₄, lanes marked F or N, respectively. All cultures in (B) were incubated in darkness, non supplemented cultures are marked C, for cultures supplemented with fructose or sucrose, lanes are marked F and S, respectively.

double, triple and quadruple heterocysts were also observed (Fig. 3A). Multiple heterocysts were mostly observed at the end of the filaments but occasionally in their middle. Cultures grown in light would occasionally show filaments with double heterocyst at the ends but not triple and quadruple heterocysts (data not shown). Cultures supplemented with sucrose and dextrin were composed of many filaments with generally smaller and more square vegetative cells than the

fructose incubated. No filaments with multiple heterocysts were detected and many filaments lacked heterocysts (Fig. 3B and C). The morphology of the filaments in the control cultures appeared degraded (data not shown) and the culture media was colored pink by phycoerythrin leakage from the cells.

Expression of hetR

To further investigate the physiological response of exogenous supplied carbohydrates in *Nostoc* PCC 9229, the expression of the heterocyst regulatory gene *hetR* was examined in cultures treated with fructose, sucrose, light and ammonia (Fig. 4). Light only and in combination with fructose resulted in two transcripts 1.4 kb and 2.0 kb in size (Fig. 5A). Cultures supplemented with light and ammonia, which inhibit the formation of heterocyst, showed a single transcript of 1.4 kb in size. In spite of using a higher RNA concentration (30 μ g versus 20 μ g) on the blots with RNA from cultures kept in darkness (Fig. 5B), the *hetR* expression was not detectable in the control, unsupplemented, samples kept in darkness. In contrast, incubating with fructose resulted in a strongly expressed transcript, 1.4 kb in size, and addition of sucrose resulted in single transcript of the same size but much weaker expressed. The transcript of 1.4 kb was slightly faster migrating in the gel than a transcript detectable at lower stringency washing (data not shown). This larger transcript most likely corresponded to the small subunit ribosomal RNA.

4. Discussion

In this study we have focused on the effects of different carbohydrates on the nitrogen fixation and heterocyst differentiation of the intracellular micro-symbiont of *Gunnera monoica*, *Nostoc* PCC 9229. The data presented, indicate that fructose and possibly glucose are the prime candidates for being the carbon compound transferred from the host *Gunnera* to monitor the nitrogen fixation of the intracellular *Nostoc* in darkness. The carbohydrate-rich *Gunnera* mucilage had no detectable effect on nitrogen-fixing capabilities of *Nostoc* PCC 9229. This is perhaps not surprising in view of the fact that the mucilage induces hormogonia formation (Rasmussen et al., 1994) which are filaments that do not fix nitrogen.

Overall, fructose maintained and stimulated the highest dark and light nitrogen fixation activity. As carbohydrates are actively transported (and metabolised) into cyanobacteria (Smith, 1982) it may be that different carbohydrate transporters vary in efficiency. The preference of either glucose or fructose for nitrogen fixation in darkness vary in different *Nostoc* strains as been shown using 18 *Nostoc* strains (Huang and Chow, 1988). In our case, dark

nitrogen fixation by free-living cultures showed rapid enhancement with light which is consistent with the view that *Nostoc* has a light stimulated nitrogen fixation activity (Steinberg and Meeks, 1991). The rapid response to light is also observed when symbiotic stem tissues are isolated from *Gunnera manicata* (J. Wouters, unpublished observations).

The potential for using externally supplied mono-saccharides for nitrogen fixation was also displayed in light and has been observed for another *Gunnera* isolate (Silvester and McNamara, 1976) as well as an *Anabaena* isolated from *Azolla* (Rozen et al., 1986). In addition, there was a relatively rapid response (90 min) to the mono-saccharide in darkness, therefore the ability to grow heterotrophically on carbohydrates may be an advantage for cyanobacteria in environments where these sugars are present. Further studies on the utilization of carbohydrates for nitrogen-fixation is needed to clarify this.

After 4 weeks in darkness, the sucrose and dextrin supplemented *Nostoc* PCC 9229 cultures were capable of nitrogen fixation, but only after 24 h exposure to light. This is possibly because new heterocysts had to form before fixation could occur. As a comparison, *Anabaena* PCC 7120 takes about 24 h to form a functional heterocyst (Wolk et al., 1994). As shown here for *Nostoc* PCC 9229, many free-living heterotrophic *Nostoc* strains cannot use sucrose for nitrogen-fixation in the dark (Huang and Chow, 1988) and this is also the case for an *Azolla* isolate (Tel-Or and Sadovsky, 1982). However, the more complex sugars, sucrose and dextrin might be used for cell survival when *Nostoc* PCC 9229 is subjected to darkness. Alternatively, these sugars remain unmetabolised and serve as an osmolyte, thus keeping the cells intact.

The multiple heterocysts that were observed in the fructose supplemented cultures may be the result of an increased carbon to nitrogen ratio in the cells of *Nostoc* PCC 9229. A high heterocyst frequency can also be induced by fructose in cultures of *Anabaena azollae* (Rozen et al., 1986). Multiple heterocysts found under symbiotic conditions are suggested to be the result of either the host acting as an efficient nitrogen sink or the chemoheterotrophic conditions (Bergman et al., 1996). The data presented here support the latter.

The expression of *hetR* observed in light were in consistency with those presented for *Anabaena* PCC 7120 (Buikema and Haselkorn, 1991). It is known to be an autoregulated serine protease (Black et al., 1993; Zhou et al., 1998). However, the detailed function of the *hetR* gene is not yet known, although necessary for heterocyst formation it is also present in non-heterocystous filamentous cyanobacteria (Janson et al., 1998) and it has been implicated to be important for development of akinetes (Leganes et al., 1994). A function in the sensing of the carbon to nitrogen ratio have also been suggested (Buikema and Haselkorn, 1991). Since we here increased the carbon to nitrogen ratio our data suggest that *hetR* is directly, or indirectly, involved in carbon metabolism as the expression patterns could be altered by addition of fructose.

In plants, sucrose is not directly imported into the amyloplast for starch synthesis but is first degraded into fructose and glucose (Hill and Smith, 1991; Schott et al., 1995). Amyloplasts are common in the surrounding non-infected *Gunnera* stem cells (Bergman et al., 1992). The effects of fructose and glucose seen here is intriguing, because in plants these hexoses are involved in a cascade of cell signaling processes and pathways such as starch synthesis. It is therefore possible that *Nostoc* is perceived by *Gunnera* as a "pseudo-amyloplast" and is regulated by these same pathways.

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