

The *Nostoc-Gunnera* symbiosis: carbon fixation and translocation

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The in vitro specific activity of ribulose-1,5-bisphosphate carboxylase (Rubisco; EC 4.1.1.39) and the dark and light in vivo CO_2 fixation activities were determined in the cyanobiont of *Gunnera*. Compared to the free-living isolate *Nostoc* PCC 9231, the in vitro Rubisco activity was high, while the in vivo CO_2 fixation was very low. Light did not significantly influence CO_2 fixation if the cyanobiont was left in the sliced *Gunnera* tissues, while a small light stimulation was found for CO_2 fixation of the freshly-isolated cyanobiont. The adjacent non-infected *Gunnera* tissue showed a very low CO_2 fixation. A rapid translocation of fixed $^{14}\text{CO}_2$ from leaves towards apical parts of the plant was apparent, in particular to the symbiotic tissue. The ^{14}C label appeared mainly in soluble form in this tissue and was rapidly catabolised as shown by ^{14}C chase experiments. Also, short-term experiments revealed that maximum ^{14}C accumulation occurred in the symbiotic tissue showing the highest rates of nitrogen fixation (Söderbäck et al. 1990), about 10–15 mm from the plant apex. The data were taken to indicate that there is a modification in the photosynthetic light reaction of the cyanobiont and that the cyanobiont lives heterotrophically in the dark on photosynthate rapidly delivered from nearby leaves of the host plant.

Key words – Carbon fixation, carbon translocation, cyanobacteria, *Gunnera*, *Nostoc*, photosynthesis, Rubisco, symbiosis.

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Introduction

Cyanobacteria develop symbiotic interactions with a variety of eukaryotic plants ranging from algae to angiosperms (Rai 1990), though *Gunnera* is the only angiosperm genus represented in this group. In all *Gunnera* species examined *Nostoc*, a filamentous and heterocystous cyanobacterium, is the cyanobiont. A unique feature of this plant-cyanobacterial symbiosis is the intracellular location of the cyanobiont (Bonnett 1990, Bergman et al. 1992). Silvester and Smith (1969), using $^{15}\text{N}_2$, demonstrated that nitrogen fixed by the cyanobiont is translocated to the host plant. The symbiotic *Gunnera* tissue is a highly specialised structure. It develops as a glandular tissue in very young apical parts of the plant stem; cyanobacteria are not required to induce its formation (Bonnett 1990, Johansson and Bergman 1992).

A distinct developmental pattern of nitrogen fixation, heterocyst frequencies and nitrogenase protein, the en-

zyme responsible for the reduction of N_2 to NH_3 , along the stem of *G. magellanica* has been demonstrated (Söderbäck et al. 1990). From these studies it was concluded that the nitrogenase activity is dependent on the status of the leaf being in close proximity to the symbiotic tissue. At the stage when a leaf senesced, nitrogen fixation in nearby symbiotic tissue ceased although the nitrogenase protein was largely retained. This pointed towards a dependence of the cyanobiont nitrogenase activity on delivery of photosynthates from the host. Certain free-living *Nostoc* spp. are able to utilise a variety of carbon compounds for heterotrophic growth (Smith 1982). Heterotrophic capacity of *Nostoc* isolated from *Gunnera* was also previously demonstrated (Harder 1917). The isolates could use some saccharides, primarily hexoses, and even a few polysaccharides, as carbon source. Since the cyanobiont in *Gunnera* resides intracellularly within the plant stem, out of reach of light, it is likely to be dependent on an efficient delivery of carbon from the host. In addition, a light-dependent

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oxygen evolution was not detected, nor were phycobiliproteins, in the cyanobiont of *G. albocarpa* (Silvester 1976). In contrast, the cyanobiont in *G. magellanica* contains similar levels of the light-harvesting pigments chlorophyll *a* and phycobiliproteins, and the enzyme Rubisco involved in photosynthetic carbon fixation, as do free-living isolates (Söderbäck et al. 1990, Söderbäck and Bergman 1992). The light dependent CO₂-fixation is greatly diminished in the cyanobacteria living in symbiosis with the hornwort *Anthoceros* (Steinberg and Meeks 1989) and in the cycads *Cycas circinalis* (Perraju et al. 1986) and *Macrozamia riedlei* (Lindblad et al. 1991). On the other hand, the cyanobiont of the water fern *Azolla* retains photosynthetic activity in the symbiotic state (Ray et al. 1979), but since sucrose is translocated from the host to the cyanobiont (Peters et al. 1985) and since the cyanobionts part of the symbiosis total CO₂-fixation activity is very low (Kaplan and Peters 1988), a photoheterotrophic type of metabolism in the cyanobiont was suggested (Braun-Howland and Nierwicki-Bauer 1990).

Here we report on diminished carbon fixation abilities and activities of the cyanobionts in *Gunnera*, and of translocation of photosynthates from plant to cyanobiont. Data were obtained from two selected *Gunnera* species, *G. magellanica* and *G. chilensis*.

Abbreviations – PVPP, polyvinylpolypyrrolidone; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); RuBP, ribulose-1,5-bisphosphate.

Materials and methods

Plant material, cyanobacterial strains, and growth conditions

Gunnera chilensis Lam. and *Gunnera magellanica* Lam. plantlets were grown in a greenhouse. Before use, the plants were acclimatised for 4 weeks in a growth chamber. The plants were grown on vermiculite, extensively watered with MS medium (Murashige and Skoog 1962) lacking combined nitrogen, at a RH of 70%, a temperature of 20°C, and a photon flux density of 120 µmol m⁻² s⁻¹ (General Electric, Power Groove de Luxe Cool White F96 PG 17).

Nostoc strain PCC 9231 was isolated from *G. chilensis*, and grown autotrophically in BG11₀ medium (Stanier et al. 1971) in continuously stirred batch culture at 50 µmol m⁻² s⁻¹ and 20°C.

Cell preparations

Nostoc PCC 9231 was harvested at late log phase. For the in vitro Rubisco activity assays, the cells were washed twice in cold TEMBM buffer (20 mM Tris, 1 mM EDTA, 10 mM MgCl₂, 50 mM NaHCO₃, 40 mM β-mercaptoethanol, pH 8.0) by centrifugation at 2500 g for 3 min. For the in vivo assays, intact filaments were washed in BG11₀ medium with 5 mM HEPES at pH 7.0 and pelleted.

Cells from *Gunnera chilensis* were obtained from symbiotic tissue located in the apical region of the plant stem. Only those close to fresh petioles were used. The symbiotic tissue was separated from the non-symbiotic stem tissue with a scalpel and homogenised gently in cold BG11₀/HEPES buffer, with 0.5% PVPP (polyvinylpolypyrrolidone; Sigma, St Louis, MO, USA) added. The material was centrifuged at 300 g for 10 min and the supernatant filtered through 8 layers of cheese-cloth to get rid of plant cell debris. The filtrate was centrifuged at 2500 g for 3 min. The supernatant obtained represented a cytoplasmic preparation, used for analysis of the in vitro Rubisco activity of the plant cells in the symbiotic tissue. The pellet was resuspended in isolation buffer and centrifuged at 300 g for 10 min. The new pellet was discarded (plant debris) and the supernatant was centrifuged at 2500 g for 3 min. This pellet, containing freshly isolated cyanobiont, was resuspended in the isolation buffer and the washing procedure repeated 3 times.

Specific in vitro CO₂-fixation activity

The two cyanobacterial cell preparations obtained, one from the free-living isolate *Nostoc* PCC 9231 and one from the cyanobiont, were resuspended in 5 ml TEMBM buffer. Cells were broken by ultrasonication and cell-free extracts were obtained by collecting the supernatant after centrifugation at 40 000 g for 30 min at 4°C.

The Rubisco in vitro assays were performed as follows: 50 µl extract was placed in a scintillation vial. A 20-µl solution containing 50 mM NaH¹⁴CO₃ (0.5 MBq mol⁻¹; Amersham, Buckinghamshire, UK) was added, and the vial was placed in a water bath at 30°C for 2 min. The reaction was started by addition of 5 µl 11.0 mM RuBP (tetrasodium salt, Sigma, St Louis, MO, USA) in TEMBM buffer. The reaction was run for 5 to 60 min and was stopped by the addition of 0.425 ml 25% TCA. In controls the RuBP substrate was omitted. Unincorporated ¹⁴CO₂ was evaporated overnight, whereafter scintillation liquid was added (Emulsifier-Safe, Packard, Groningen, Holland) and scintillation counting performed (SL 30, Liquid Scintillation Spectrometer, Intertechnique, Plaisir, France).

In vivo CO₂-fixation activity

Cells of the free-living isolate *Nostoc* PCC 9231 and the cyanobiont from *G. chilensis* were prepared as described above. The final pellet was resuspended in 10 ml BG11₀/HEPES buffer. Also, thin slices (~0.5 mm thick) of symbiotic and nearby non-symbiotic tissues were obtained from *G. chilensis* and *G. magellanica* after dissecting the tissues with a scalpel. The sliced tissues were weighed (fresh weight) and placed in 1 ml BG11₀/HEPES buffer, pH 8.0, with H¹⁴CO₃⁻ added (0.2 MBq mol⁻¹) in 5 ml glass tubes. The tubes were sealed

with rubber stoppers. Eight replications, of which 4 were covered with aluminium foil, were incubated horizontally at $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 20°C on a vertical shaker. The reaction was stopped by pelleting the cells at 2500 g for 3 min and resuspension in 2 ml 25% TCA. The preparations were transferred to scintillation vials left open overnight to evaporate unincorporated $^{14}\text{CO}_2$. Scintillation liquid was added (20 ml) and scintillation counting performed as above.

Translocation experiments on *Gunnera magellanica*

Leaf carbon fixation and translocation

The second expanded leaf of intact *G. magellanica* plantlets was incubated for 1 h in $^{14}\text{CO}_2$. This was done by inserting the leaf through an opening, 1 cm in diameter, in a flat 250 ml clear plastic (polycarbonate) container with a rubber seal at top. The opening was sealed with Blu-Tack (Emhart-Bostik, Leicester, UK). Plants were chosen so that a stolon emerged from the upper part of the plant as illustrated in Fig. 2. Just below the rubber seal a tube containing 10 µl 40 mM $\text{H}^{14}\text{CO}_3^-$ solution (1.96 TBq mol $^{-1}$) was glued with Blu-Tack on to the wall of the plastic container. $^{14}\text{CO}_2$ was generated by injecting, with a syringe, 50 µl 1 M HCl into the reaction tube through the rubber stopper. The plants were incubated for 60 min at $120 \mu\text{mol m}^{-2} \text{s}^{-1}$, RH 70% and 20°C. In a second experiment, three apical leaves were incubated in $^{14}\text{CO}_2$ as described above and as illustrated in Fig. 2E.

After exposure to $^{14}\text{CO}_2$, tissues were analysed either immediately or after 12 h of chase in light. Different plant parts were separated as shown in Fig. 2, and treated in either of two ways:

(1) Separation of soluble and insoluble cell material. The tissue was homogenised with a glass homogeniser in TE buffer (50 mM Tris, 5 mM EDTA, pH 7.0). Fractionation was performed in a microcentrifuge at 13 000 g for 30 min, the supernatant representing the soluble fraction and the pellet the insoluble fraction.

(2) Separation of symbiotic and non-symbiotic tissues. The tissues were separated with a scalpel, the fresh weight determined and the tissue homogenised with a glass homogeniser.

Scintillation liquid (Packard, see above) was added (20 ml) to each sample and ^{14}C label determined by scintillation counting as above either immediately after the $^{14}\text{CO}_2$ incubation or after 12 h of chase in light.

Stem tissue carbon fixation and translocation

Roots and leaves were separated from stems of *G. magellanica*. The stripped stems were placed in clear 15 ml polycarbonate tubes with a rubber seal. Labelled $^{14}\text{CO}_2$ (150 kBq), generated as described above, was added and the stems were incubated for 15 min as described above. The stems were then divided into symbiotic and non-symbiotic tissues, their fresh weight determined, and the tissues homogenised in a glass homo-

geniser. Scintillation liquid was added (20 ml), and scintillation counting performed as above.

Protein

Protein determinations were performed with the Bio-Rad Protein Assay kit (Bio-Rad, München, Germany).

Chlorophyll

The cyanobacterial cell preparations were pelleted by centrifugation (2500 g for 3 min) and resuspended in 2 ml 80% cold acetone. Plant tissues were gently homogenised in TE buffer, and the homogenates filtered through glassfiber filters (Munktell, Kebo, Stockholm, Sweden). The filters were placed in 2 ml 80% cold acetone. Vials with suspension or filter in acetone were covered with aluminium foil and incubated at 4°C for 2 h, whereafter chlorophyll content was determined according to Harborne (1973).

Results

The symbiotic tissue

Symbiotic tissues of the *Gunnera* species used in these studies are embedded in very compact plant stem tissue. In the larger species, *G. chilensis*, the symbiotic tissue is located rather deep (1–2 cm) in the thick stem, which is covered by a layer of scales. No light can penetrate this tissue. The smaller species, *G. magellanica*, has a more smooth green stem surface, 7–10 mm in diameter and lacks the thick layer of scales. The symbiotic tissue is located below the chlorophyll-containing outer cell layers, embedded in a non-chlorophyllous tissue to which probably no light will penetrate.

Cell preparation from the symbiotic tissues

To be able to investigate the activity of the cyanobiont it was necessary to develop a procedure to separate the cyanobiont from the remainder of the *Gunnera* symbiotic tissues. By very gentle grinding, filtering through a number of layers of cheesecloth followed by extensive washing, it was possible to generate *Nostoc* preparations void of contaminating plant tissue. Addition of osmoticum proved to be of no significance for the results obtained.

However, the method was tedious and only small fractions of the symbiotic *Nostoc* populations were obtained. This made isolation of the cyanobiont from the small *G. magellanica* more or less impossible, and only symbiotic tissue from the larger *G. chilensis* plants was prepared.

In vivo carbon fixation

Under the conditions used, the free-living *Gunnera* isolate *Nostoc* PCC 9231 showed an in vivo CO_2 -fixation

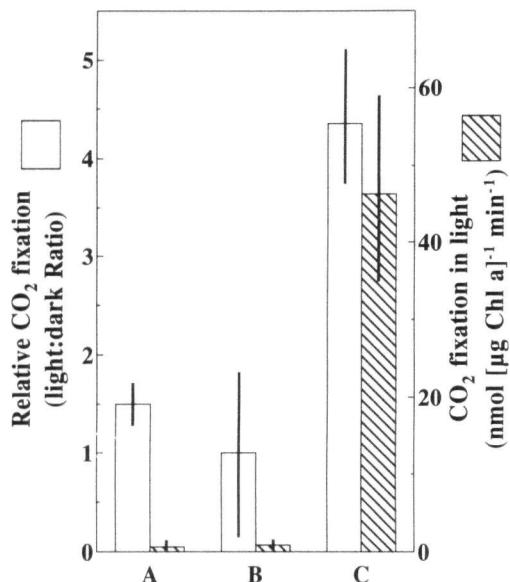


Fig. 1. In vivo $^{14}\text{CO}_2$ fixation activity in (A) freshly isolated cyanobiont from *Gunnera chilensis* ($n = 4$), (B) intact (sliced) *G. chilensis* symbiotic tissue ($n = 4$), and (C) the free-living *Nostoc* PCC 9231. (Thin bars = SD, $n = 6$).

rate in light of $46 \text{ nmol} (\mu\text{g Chl } a)^{-1} \text{ min}^{-1}$. The rates in the freshly isolated cyanobiont from *G. chilensis* and in the intact (sliced) symbiotic tissues of *G. chilensis* were similar. However, activities were less than 2% of that detected in the free-living *Nostoc* PCC 9231 (Fig. 1). In darkness, the in vivo CO_2 -fixation rate of the free-living *Nostoc* PCC 9231 decreased to approximately 23% of that found in light. In intact (sliced) symbiotic tissues of *G. chilensis* the dark CO_2 -fixation rate was similar to that in light, while in the freshly isolated cyanobiont 70% of the activity remained (Fig. 1).

The in vivo CO_2 -fixation in symbiotic and non-symbiotic stem tissues were compared in *G. chilensis* and *G. magellanica* (data not shown). Both tissue types showed similar levels of fixation activity in both plants. The activity noted was very low compared to the outer chlorophyllous stem (*G. magellanica*) and leaf tissues (*G. magellanica* and *G. chilensis*).

In vitro Rubisco assay

The in vitro Rubisco activities of the free-living isolate *Nostoc* PCC 9231, of the freshly isolated cyanobiont of *G. chilensis*, and of the intact (sliced) symbiotic tissues,

Tab. 1. Specific in vitro Rubisco activity in cell extracts of the cyanobiont, the free-living *Nostoc* PCC 9231 and plant cells from symbiotic tissue of *G. chilensis*.

Material	CO_2 fixed, nmol (mg protein) ⁻¹ min^{-1} (SD)
Freshly isolated cyanobiont	210 ± 120 ($n = 4$)
Free-living isolate (<i>Nostoc</i> PCC 9231)	132 ± 31 ($n = 12$)
Plant cells in symbiotic tissue	21 ± 21 ($n = 4$)

are shown in Tab. 1. The cyanobiont showed an in vitro Rubisco activity being 10 and 1.6 times higher than that of the *Gunnera* symbiotic cells and that of the free-living isolate *Nostoc* PCC 9231, respectively.

Translocation experiments

After exposing one specific leaf (the second from apex) on the *G. magellanica* plantlet to $^{14}\text{CO}_2$, the results of ^{14}C -label were analysed in two ways. First, the relative ^{14}C in the soluble and insoluble fractions of various plant parts, expressed as % of total plant ^{14}C label (Fig. 2A,B) was examined. After 60 min incubation with CO_2 (Fig. 2A), the majority of the label was in the soluble fraction of the incubated leaf, but substantial label was also found in the stem part connected to the incubated leaf (22% of total plant ^{14}C) as well as in the adjacent apical stem part (11% of total plant ^{14}C). As in the incubated leaf, most of the label was in the soluble fraction. A relatively high label, 11% and 7%, respectively, was also found in roots connected to the stem part with the incubated leaf and the stem part apically located. Again, the label appeared almost exclusively in the soluble fractions. Other parts of the plant showed low relative label intensities. After a 12-h chase in light (Fig. 2B) the relative label of the incubated leaf had decreased considerably, now being 14% of plant total label, and the ^{14}C label had spread to more remote plant parts. Now, the ^{14}C was mainly localised in plant parts apical to the incubated leaf. The proportion of ^{14}C in insoluble fractions increased, especially in roots and in the stolon apex. The fraction of relative ^{14}C label in stem tissues decreased after the chase.

Second, the ^{14}C per unit fresh weight, also on a relative basis (percentage of the highest Bq value of a plant tissue), after separation of symbiotic and non-symbiotic plant tissues was examined (Fig. 2C,D). After 60 min exposure to $^{14}\text{CO}_2$, the highest amount of ^{14}C label was found in the incubated leaf. Substantial label was found in the symbiotic tissue of the stem connected to the incubated leaf or located apically to this, 25% and 26%, respectively. This should be compared to 10% and 6%, respectively, in the non-symbiotic tissues of the same stem segments. Also the roots connected to the incubated leaf showed a relatively high level, 29% of the label in the incubated leaf. In the chase experiment, the label in the symbiotic tissue increased substantially, in particular in the stem part connected to the incubated leaf. This tissue was now labelled 10 times more intensively than the non-symbiotic tissue (Fig. 2D). Overall, stem parts at or apically located to the incubated leaf had a higher ^{14}C -label in the symbiotic compared to non-symbiotic tissue. An exception was the plant apex, where the non-symbiotic tissue was almost as extensively labelled as the symbiotic tissue, 50% and 61%, respectively.

In another translocation experiment, 3 leaves were incubated in $^{14}\text{CO}_2$ followed by a 12-h chase. The distri-

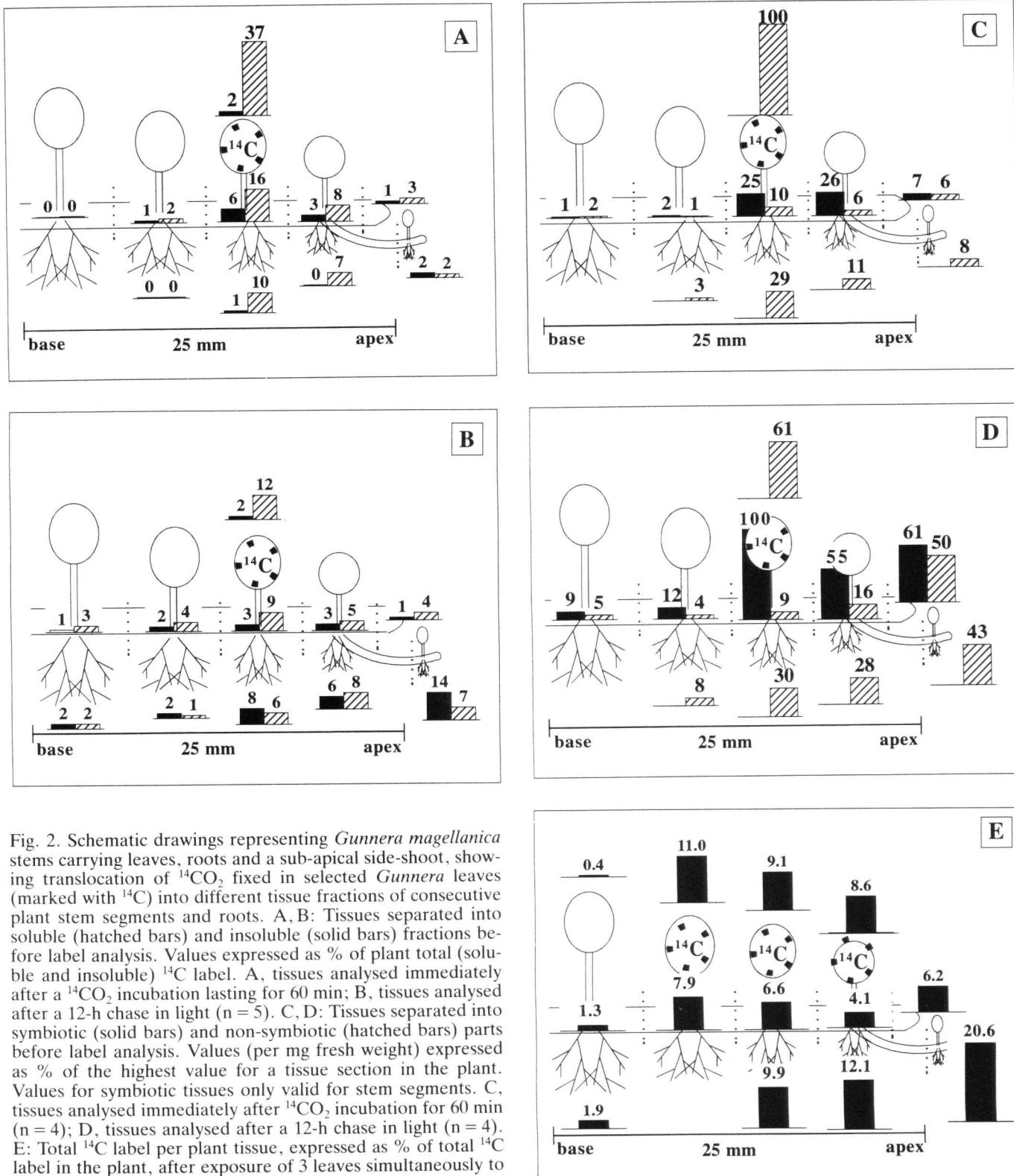


Fig. 2. Schematic drawings representing *Gunnera magellanica* stems carrying leaves, roots and a sub-apical side-shoot, showing translocation of $^{14}\text{CO}_2$ fixed in selected *Gunnera* leaves (marked with ^{14}C) into different tissue fractions of consecutive plant stem segments and roots. A, B: Tissues separated into soluble (hatched bars) and insoluble (solid bars) fractions before label analysis. Values expressed as % of plant total (soluble and insoluble) ^{14}C label. A, tissues analysed immediately after a $^{14}\text{CO}_2$ incubation lasting for 60 min; B, tissues analysed after a 12-h chase in light ($n=5$). C, D: Tissues separated into symbiotic (solid bars) and non-symbiotic (hatched bars) parts before label analysis. Values (per mg fresh weight) expressed as % of the highest value for a tissue section in the plant. Values for symbiotic tissues only valid for stem segments. C, tissues analysed immediately after $^{14}\text{CO}_2$ incubation for 60 min ($n=4$); D, tissues analysed after a 12-h chase in light ($n=4$). E: Total ^{14}C label per plant tissue, expressed as % of total ^{14}C label in the plant, after exposure of 3 leaves simultaneously to $^{14}\text{CO}_2$ for 60 min ($n=1$).

bution of label in different plant parts, expressed as % of total plant ^{14}C , was determined (Fig. 2E). This time a high label was detected in stem parts and roots in the vicinity of the incubated leaves and towards the apical region. The highest ^{14}C label was found in the stolon apex (20.6% of total plant labelling). The basal parts of

the stem with leaves that were not $^{14}\text{CO}_2$ -incubated showed little or no ^{14}C label.

To follow carbon translocation to symbiotic tissues in a shorter time sequence, i.e. without the label having to pass via leaves, the photosynthetic activity in the outer chlorophyllous stem tissue of *G. magellanica* were used.

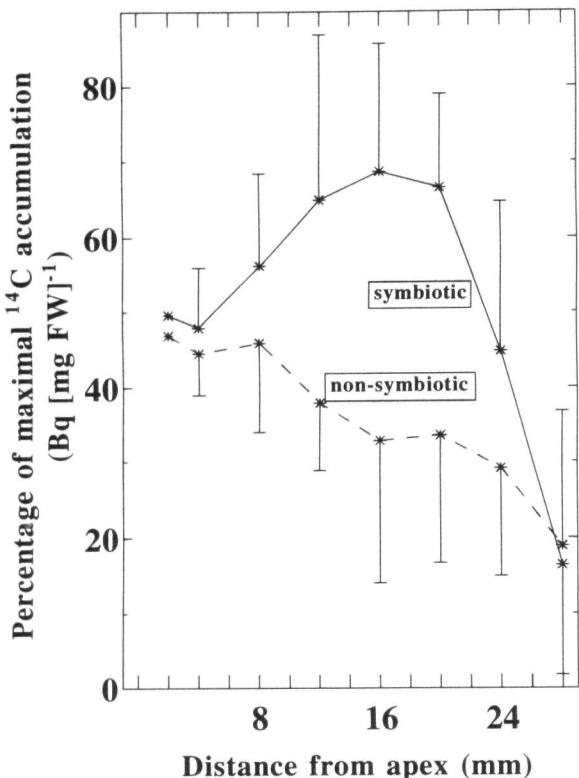


Fig. 3. ^{14}C accumulation per mg FW in symbiotic (solid line) and non-symbiotic (hatched line) tissues in different parts along *Gunnera magellanica* stems stripped of leaves and incubated for 15 min in $^{14}\text{CO}_2$. Expressed as % of the highest value in a tissue in the plant. (Bars = SD, n = 12).

Stems were stripped of leaves and roots and incubated in $^{14}\text{CO}_2$ for 15 min and immediately analysed for total ^{14}C labelling in symbiotic and non-symbiotic tissues. The relative ^{14}C distribution per mg fresh weight in the plant is shown in Fig. 3. Both tissues assimilated similar amounts of carbon in the most apical part of the plant. The symbiotic tissues accumulated increasing quantities of ^{14}C label along the plant until a maximal value was reached about 15 mm from the apex, whereafter the label declined down to almost zero in the basal part of the plant. The non-symbiotic tissues showed a continuous decline in accumulated ^{14}C -label per mg fresh weight along the whole stem.

Discussion

We have previously shown that pigments, such as chlorophyll *a* (Söderbäck et al. 1990) and phycobiliproteins, as well as Rubisco (Söderbäck and Bergman 1992) are present in the cyanobionts of *G. magellanica* in amounts equivalent to that of free-living photoautotrophically grown cyanobacteria. Similar results were found for the larger species *G. chilensis* (E. Söderbäck, unpublished). These findings, together with the low in vivo and high in vitro CO_2 fixation activities in the cyanobiont of both *G. magellanica* and *G. chilensis* obtained, demonstrate that the photosynthetic machinery of the cyanobionts to a major extent is intact although not functional. This is in

contrast to what was found in the *Azolla-Anabaena* relationship, where the cyanobiont *in vivo* showed very low CO_2 fixation activity while the freshly isolated cyanobiont showed activities comparable to free-living isolates (Kaplan and Peters 1988). The dysfunctionality in the *Nostoc-Gunnera* association is not localised to Rubisco, which shows appreciable in vitro activities in the cyanobiont of *G. chilensis* (Tab. 1). Rather, the activity in the cyanobiont was unexpectedly high, being approximately 1.6 times higher than that of the free-living *Nostoc* isolate. The cause of this is not known. The data for *Gunnera* contrast to those obtained for the *Nostoc-Anthoceros* symbiosis (Steinberg and Meeks 1989) in which the in vitro Rubisco activity of the cyanobiont was almost 10-fold lower than that of the free-living isolate used for comparison. As the Rubisco protein levels were similar in the two organisms, a post-translational modification of Rubisco was proposed (Steinberg and Meeks 1989). This type of regulation is apparently not the case in the cyanobiont of *G. chilensis*.

On the other hand, the results obtained suggest that down-regulation does occur within the photosynthetic machinery of the cyanobiont in *Gunnera*. This is manifested as a specific constraint in the light dependent photosynthetic electron transport chain. Such a conclusion is based on the fact that the light stimulation seen in the CO_2 fixation activity of the free-living *Nostoc* (more than 4-fold) is lacking in the *Gunnera* cyanobiont, in particular when the cyanobiont is retained in the intact (sliced) *Gunnera* tissue (Fig. 1). The low but definite light stimulation seen in CO_2 fixation of the freshly isolated cyanobiont, which probably is due to a better light exposure of these cells, may however point to the retention of a low level electron transport capacity. Likewise, light did not stimulate the *in vivo* CO_2 fixation of freshly isolated cyanobionts of cycads (Lindblad et al. 1991). The cyanobacterium is also likely to live heterotrophically in the latter symbiosis, dwelling in coralloid roots of the plants (Lindblad and Bergman 1990).

In addition, the dark *in vivo* CO_2 fixation activities of the cyanobionts were always a minor fraction of the activity detected in dark-incubated free-living *Nostoc* (Fig. 1). This was the case both when the cyanobiont was freshly isolated and when still in the intact (sliced) symbiotic tissue. This is probably not due to a permanent modification but may rather depend on the fact that the cyanobiont lives heterotrophically, being in darkness inside the *Gunnera* stem tissue and hence dependent on carbohydrates from the plant. Although there are anatomical differences in the two *Gunnera* species examined, with for instance chlorophyll being present in outer layers of the stems of *G. magellanica*, the cyanobionts are in both cases always embedded in non-photosynthetic stem tissue into which no light seems to penetrate.

The fast translocation of a high proportion of the fixed carbon towards the apical regions of *Gunnera*, containing the most active symbiotic tissue (Söderbäck

et al. 1990), and to the symbiotic tissue as such (Fig. 2A,C), shows that this tissue and the cyanobiont are indeed major sinks for *Gunnera* photosynthates. Additional arguments for such a conclusion are the fast turnover rates seen in the symbiotic tissue in the chase experiments (Fig. 2A,B,E) and the high proportion of soluble carbon, before and after the chase period, in this tissue (Fig. 2A,B). These probably represent a pool of easily catabolised carbohydrates for the cyanobiont.

The translocation of carbohydrates from the leaves (source) to the symbiotic tissue (sink) must take place via the polystelic vascular tissue typical of *Gunnera* plants (Batham 1943), interconnecting the source and the sink. A high number of such steles are always seen in close proximity to the symbiotic tissue as are non-infected cells with a high frequency of starch containing amyloplasts (Bergman et al. 1992). A heterotrophic mode of life may also be one of the explanations for the high frequency of heterocysts found in *Gunnera* symbioses (Silvester 1976, Söderbäck et al. 1990). If fed with appropriate carbohydrates from the host, the normally photosynthetically active vegetative cells will not be as vitally important.

It may be deduced that senescence of a leaf will cause severe carbon starvation symptoms in nearby symbiotic tissues. This in turn will explain the previously found correlation between leaf senescence and drop in nitrogenase activity (Söderbäck et al. 1990). The translocation of carbon into the symbiotic tissue was particularly pronounced in the intermediately aged part of the *Gunnera* stem, i.e. 10–15 mm from the *Gunnera* apex (more than double the level transported into the non-symbiotic tissue; Fig. 3). As this is the stem region in which the peak nitrogenase activity occurs (Söderbäck et al. 1990) the nitrogen fixation process is obviously closely dependent on photosynthate catabolism for supply of necessary energy and carbon-skeletons. The light stimulated nitrogen fixation detected in *G. albocarpa* (Silvester 1976), a species which like *G. magellanica* contains chlorophyll in the outer layers of the stem, may therefore reflect a fast translocation of photosynthate from these layers rather than a direct light effect on the cyanobiont. A dependence on plant photosynthates to sustain nitrogen fixation is also known from defoliation experiments in other angiosperm symbioses involving *Rhizobium* (Culvenor and Simpson 1990) and *Frankia* (Vikman 1990), and is probably the case in all cyanobacterial-plant symbioses except for in the two-component lichens where the cyanobiont in addition supports the fungus with carbohydrates (see Rai 1990).

In conclusion, although key photosynthetic pigments and proteins are present in the cyanobiont of *Gunnera*, CO₂ fixation activities in dark and light are very low. The latter is not due to any modification of the Rubisco protein but rather to a non-functional photosynthetic electron transport machinery. It is also obvious that the cyanobiont lives heterotrophically on *Gunnera*-produced photosynthates as these are efficiently trans-

ported in a soluble form to the cyanobiont. This is further manifested in the finding that the maxima in delivery of fixed carbon and nitrogen fixation activity (Söderbäck et al. 1990) coincide at about 10–15 mm from the *G. magellanica* apex.

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