Fructose-Enhanced Development and Growth of the N_2-Fixing Cyanobiont *Anabaena azollae*

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Fructose supported the heterotrophic growth of the cyanobiont *Anabaena azollae,* isolated from the water fern *Azolla filiculoides,* and also enhanced its growth in the light by 2–3-fold. Fructose was taken up at a high rate in the light and in the dark, in an energy-dependent reaction. The photosynthetic and respiratory activities of the fructose grown cells were modified: O_2 evolution *in vivo* was decreased by 40%, while PS I activity and dark respiration were 2–3-fold higher than in autotrophically grown cells. These changes were accompanied by 2–3-fold increase in heterocyst differentiation and by a 4-fold stimulation of nitrogenase activity.

Introduction

The symbiotic relationship between the water fern *Azolla* and the N_2-fixing cyanobiont *Anabaena azollae* has been extensively studied, and it is generally accepted that the cyanobiont is supported by photosynthates provided by the host [1]. Extracts of the host fern *Azolla* were found to contain high concentrations of sucrose, fructose and glucose, which are assumed to be available to the cyanobiont in the leaf cavity [2, 3]. Fructose was previously observed to support growth in *Anabaena azollae* isolated from *Azolla caroliniana* [4] and *Azolla filiculoides [5]. The ability to take up and metabolize exogenous sugars, and utilize them as major carbon sources for energy supply and growth, is therefore considered a prerequisite for the symbiotic relationship of Anabaena azollae* with its host *Azolla*.

Photosynthates supplied by the host might be expected to modify the energy metabolism of the cyanobiont, increasing respiratory activity and decreasing photosynthesis and altogether inducing a heterotrophic-growth pattern. Currently there is very little known on the effects of photosynthates provided by *Azolla* on the respiratory and photosynthetic activities of the cyanobiont. There is also practically no information on the photosynthetic activity of cyanobacteria under conditions of sugar supported growth, either in the light or in the dark, and only limited information on respiratory activity under such conditions [6, 7].

Modifications induced by exogenous sugars in the metabolism of the cyanobiont, are also expected to include enhancement of nitrogen fixation, which is the main contribution of the cyanobiont to the symbiosis. Enhancement of nitrogenase activity by fructose and sucrose was indeed reported for *Anabaena azollae,* isolated from *Azolla caroliniana, Azolla filiculoides* and *Azolla pinnata* [8–10]. Growth of *Anabaena variabilis* in the presence of fructose, was found to involve accumulation of glycogen. It was suggested that glycogen accumulation is necessary to stabilize nitrogenase activity [11, 12]. An increased nitrogenase activity accompanied by glycogen accumulation was also found in fructose-grown *Anabaena azollae* [5].

In experiments described in this communication, we tested the effects of fructose on growth and development of *A. azollae* in the dark and in the light, and analyzed the responses to fructose of N_2-fixation, photosynthesis and respiration in these cells. Growth of *Anabaena azollae* in the presence of fructose induced major modifications in its photosynthetic and respiratory activities, adapting it to heterotrophic growth and to its role as the nitrogen fixing partner in the symbiosis.

Abbreviations: CCCP, (carbonylcyanide m-chlorophenylhydrazone); DCMU, [3-(3,4-dichlorophenyl)-1,1-dimethylurea].

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Materials and Methods

Organism and cultures

Anabaena azollae was isolated in our laboratory from the water fern *Azolla filiculoides* [13]. The cul-

ture was routinely grown as described by Tel-Or and Sandovsky [8]. Cells from two week old cultures were transferred to 250 ml Erlenmeyer flasks containing BG-11 medium [14], and were grown in the dark or in the light (I = 5 watt · m^{-2}), with or without added sugar, for one to two weeks.

Analytical procedures and assays

Cells of *Anabaena azollae* were collected every 24 h for analysis. Representative results from 4–6 experimental series are shown, each result being the average of duplicate or triplicate determinations. Nitrogenase activity, measured by acetylene reduction, cell dry weight and glycogen content was determined as described by Rozen *et al.* [5]. Sugar content of the medium was determined by the phenol sulfuric acid procedure [15]. Chlorophyll was determined according to Mackinney [16].

Fructose incorporation was studied with [U-^{14}C]fructose. Cells were incubated at room temperature for 15 min in BG-11 medium containing 8 mM fructose with 0.06 μCi [U-^{14}C]fructose per sample, on an illuminated shaker (I = 5 W · m^{-2}). The cells were collected and washed on a milipore filter (0.45 μm), suspended in scintillation liquid, and analyzed with a Beckman LS 7800-counter. Photosynthesis and respiration activities *in vivo* were determined by changes in oxygen concentration as measured by a Clark-type O_2 electrode (Y.S.I. 5331). Oxygen uptake in dark and evolution in light (I = 2300 μE · m^{-2} · sec^{-1}) were calculated from the slope of the curve during steady state. PS I activity was determined with protoplasts prepared by lyzozyme treatment [17]. The reaction mixture contained 5 mM sodium ascorbate, 50 μM 2,6, dichlorophenolindophenol, 50 μM methyl viologen, 1 mM NaN_3, 10 μM DCMU in 25 mM hepes pH 7.5, and protoplasts containing 20 μg Chl a · ml^{-1}. Reaction mixture for assays of PS II activity contained 25 mM Hepes buffer pH 7.5, 1 mM potassium ferricyanide, and protoplasts containing 20 μg Chl a · ml^{-1}.

Results and Discussion

Fructose was previously shown to enhance the growth of *A. azollae* in the light. Cell growth rate in batch cultures was 2–3-fold higher in the presence of 8 mM of fructose, compared to autotrophically grown cells [5]. As shown in Fig. 1, the growth pattern of the cells in the light was similar in the absence and

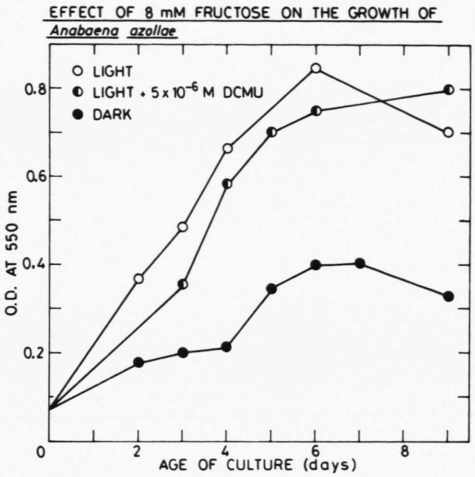

Fig. 1. Effect of 8 mM fructose on the growth of *Anabaena azollae*. Cells were grown as described in Materials and Methods. ○—○, light; ◑—◑, light +5 μM DCMU; ●—●, dark.

the presence of 5 μM DCMU. In addition, fructose-supported growth in the dark exhibited an initial lag, and the maximal cell density achieved was about half of that obtained in the light. These results seem to suggest that maximal growth in the presence of fructose depended on PS I activity, but was largely independent of PS II.

Nitrogenase activity of autotrophic cultures, as measured by acetylene reduction, which was usually about 100 μmoles ethylene · g dw^{-1} · h^{-1}, increased 2–4-fold by the presence of fructose in the growth medium. As shown in Fig. 2, nitrogenase activity in fructose-grown cells in the light was largely unaffected by DCMU, suggesting that the contribution of PS II to nitrogen fixation was insignificant under these conditions. The maximal activity in the dark was about half of that in the light, suggesting that respiration could only partially support the energetic demands of heterotrophic nitrogen fixation, and that the operation of PS I was essential for optimal activity.

Maximal nitrogenase activity was obtained between the second and fourth days of growth in the light (Fig. 2), when culture density reached about half of its maximum (0.2–0.4 mg cell dry weight/ml). The increase of nitrogenase activity coincided with a rise in frequency of heterocysts which was highest in the culture grown with 8 mM fructose. Heterocyst frequencies of 5%, 16%, 18%, and 14% were ob-

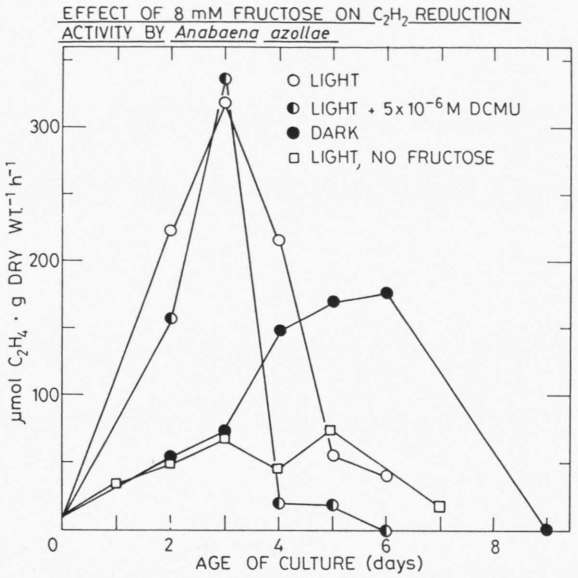

Fig. 2. Effect of 8 mM fructose on nitrogenase activity of *Anabaena azollae*. Activity of acetylene reduction was assayed as described in Materials and Methods. ○—○, light; ◐—◐, light +5 μM DCMU; ●—●, dark; □—□, light without fructose.

tained for cultures grown in the light with 0, 2.5, 8, and 16 mM fructose respectively. Acetylene reduction activity in the dark exhibited an initial lag and reached its maximum between the fourth and sixth day. The pattern of changes in frequency of heterocysts in the dark, corresponded to the pattern of changes in nitrogenase activity. The maximal heterocyst frequency in the dark was 14%. A close correlation between growth and nitrogenase activity in the dark (Fig. 1 and 2) could suggest that nitrogen fixation is the rate limiting factor for heterotrophic growth.

Fructose-grown *A. azollae* cells were previously shown to accumulate glycogen during growth in the light [5], and a similar result was obtained in the present study for fructose-grown cells in the dark. The cell glycogen content increased sharply, reaching about 20% of the cell dry weight by the second or the third day of growth, and then decreased (data not shown). The apparent correlation between the increases in glycogen content and induction of nitrogenase activity agrees with previous reports of such correlation in fructose grown *Anabaena variabilis* which might indicate stabilization of nitrogenase by glycogen [11, 12]. It was suggested that the high glycogen content could support high respiration rates, which would keep oxygen at a low concentration, and thus stabilize nitrogenase activity.

The limited effect of DCMU on fructose-supported growth and on fructose-enhanced nitrogenase activity, raised the question whether growth in the presence of fructose involved modifications of the photosynthetic apparatus resembling those occurring during the process of heterocyst differentiation. Enhanced PS I activity in heterocysts, involving an increase in number of PS I reaction-centers per cell is accompanied by a loss of PS II activity [18]. This possibility was tested by measurements of partial photosynthetic reactions in hypotonically broken protoplasts, isolated from either fructose-grown cells or autotrophically grown cells. PS I activity was measured by the light-driven electron transport from reduced dichlorophenolindophenol to methyl viologen. The specific activity of preparations from autotrophically grown cells was 200 μeq · mg $Chl^{-1} \cdot h^{-1}$, while the activity of preparations from cells grown in the light in the presence of fructose was 660 μeq · mg $Chl^{-1} \cdot h^{-1}$. PS II activity was apparently impaired during the isolation process, and no activity of electron transport from water to ferricyanide could be detected in preparations from either control or fructose grown cells. These results thus indicate that fructose induced a major increase in PS I activity but provided no information regarding the effect of fructose on PS II. An indication for fructose-induced reduction in PS II activity was found in the fact that oxygen evolution by *A. azollae* cells, grown for four days in the presence of fructose, was about 40% lower than that of cells grown autotrophically (Table I).

Table I. Photosynthetic O_2 evolution and respiratory O_2 consumption by *A. azollae*.

Cell growth conditions	O_2 uptake (dark)	O_2 evolution (light)
light grown in BG-11 medium	38	155
light grown with 8 mM fructose	50	92
dark grown with 8 mM fructose	90	92

Cells were grown for four days under the specified growth conditions. Respiratory activity was measured in the dark in the absence of exogenous sugar. Reaction rates are in μmoles O_2 · mg $Chl^{-1} \cdot hr^{-1}$. Assays were conducted with cells, containing 30–50 μg Chl a, suspended in fresh BG-11 medium.

These results correlate well with the insensitivity to DCMU of cell growth (Fig. 1) and nitrogenase activity (Fig. 2) in the presence of fructose. The 3-fold higher PS I activity may help to provide the high ATP levels required for fructose uptake and the enhanced nitrogenase activity. This increase in PS I activity induced by fructose, seems to resemble the enrichment in PS I reaction centers which takes place during differentiation of vegetative cells to heterocysts [18]. PS II activity in the latter is lost during differentiation. Preliminary studies on the effect of fructose on the ultrastructure of *A. azollae* cells have indicated a different arrangement and reduced number of thylakoids in the fructose grown cells (results not shown).

The 2–3-fold increase in growth rate in the light, induced by fructose [5], was not accompanied by a comparable increase in respiration rate, as might have been expected. The respiration of cells grown in the light in the presence of fructose was only 32% higher than that of autotrophically grown cells (Table I). It might thus be concluded that fructose enhanced growth in the light, not by serving as a substrate for respiration, but rather by enhancing PS I activity. In the dark, on the other hand, fructose was essential as a substrate, and the oxygen consumption rate was indeed two and a half times higher than that of the autotrophically grown cells. The fact that glycogen accumulates in fructose grown cells, to a similar extent in the light and in the dark, indicates that the cells contain an abundance of endogenous substrates for respiration in both cases. The increased respiratory activity of cells grown in the presence of fructose in the dark, might be due to regulatory functions of fructose which are expressed specifically in the dark, and may involve increased levels of respiratory chain components.

The fructose-supported DCMU-insensitive cell growth should involve high rates of fructose uptake, not only in the dark but also in the light. Direct measurements of the uptake of [^{14}C]fructose by autotrophically grown cells of *A. azollae* were carried out for 15 min periods. Table II shows that cells grown *in the absence of fructose,* exhibited high rates of fructose uptake, compared to previously reported measurements in other cyanobacteria [6]. Fructose uptake in the light was only partially inhibited by DCMU. Rates of uptake in the dark were also high, amounting to 60% of those measured in the light, indicating that respiration as well as photosynthesis can supply the energy requirement for fructose uptake. CCCP, which is a potent uncoupler of both oxidative and photosynthetic phosphorylation, inhibited 80 percent of the activity in the light and inhibited the activity in the dark almost completely, at a concentration of 40 μM. These results thus demonstrate the operation of a constitutive mechanism for fructose uptake in *A. azollae* cells. Fructose uptake in cells grown for four days in the presence of fructose was two fold higher than in the control, autotrophically grown cells. This may indicate further induction or activation of the uptake mechanism by its substrate. The incorporation of glucose and sucrose was also tested: the rate of glucose uptake was half of that of fructose, while sucrose uptake rate was only 15 percent of that of fructose.

Table II. Fructose uptake by *Anabaena azollae.*

Conditions	Fructose uptake [μmol·g dry wt^{-1}·hr^{-1}]
light	443 ± 33
light + 10 μM DCMU	297 ± 49
light + 40 μM CCCP	93 ± 45
dark	258 ± 75
dark + 40 μM CCCP	5 ± 3

Cells were grown autotrophically for four days in BG-11 medium. Fructose uptake was analyzed as described in Materials and Methods.

The results presented in this report demonstrate the capacity of *A. azollae* cells to incorporate and metabolize fructose for growth both in the dark and in the light. The cells possess an efficient uptake mechanism for fructose, supported by energy provided by either photosynthesis or respiration. Further enhancement of uptake activity could be induced by the presence of fructose in the growth medium. Fructose supported growth was accompanied by enhanced respiration, decreased O_2 evolution in the light but increased PS I activity. The balance of these changes should be an overall increase of the potential for ATP synthesis both in the light and in the dark.

The changes induced by the introduction of fructose into the growth medium of *A. azollae* may be analogous to the sequence of events triggered by the host photosynthates in the cyanobiont in the leaf cavity. The changes in respiratory and photosynthetic activities described above should result in reduced

dependence on self-produced photosynthates, and also in reduced oxygen concentrations. A reduction in photosynthetic CO_2 fixation was indeed reported for freshly isolated *A. azollae* [19]. Glycogen accumulation at an early phase of growth in the presence of fructose should result in an increase in the C/N ratio and support heterocysts differentiation. This, combined with the decrease in O_2 concentration, provides the conditions necessary to stimulate nitrogenase expression and activity.

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