METABOLIC AND OSMOREGULATORY ADJUSTMENTS IN THE *AZOLLA*-CYANOBACTERIA SYMBIOTIC RELATIONSHIP

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INTRODUCTION

Azolla is a fresh water fern in symbiotic relationship with nitrogen-fixing cyanobacteria (6). This system is of significance in the enrichment of many aquatic systems. The symbiotic cyanobacteria occupy the ventral cavity within the upper aerial leaves of the fern. Formation of leaf cavities does not require the presence of the cyanobacteria since they occur in plants grown under axenic conditions. The cavity contains a mucilaginous medium of unknown composition (3, 12) that is essential for survival of the cyanobacteria and for the high level of nitrogen fixation. Early studies on similar associations indicated that fixed carbon such as sucrose in the plant nourished cyanobacteria in the cavity sap (5, 8, 7, 9). In this report we attempted to define the metabolic and osmoregulatory relationship existing between the two organisms.

MATERIALS AND METHODS

Culture of *Azolla*

A. rubra (AR1) and *A. mexicana* (AX1) were grown in defined media as previously described (2). In addition, medium for cyanobacteria-free *A. mexicana* (AXF) was supplemented with 2 mM each of $CaNO_3$ and KNO_3. Photon flux density was 200 $\mu mol.m^{-2}.s^{-1}$ using cool-white fluorescent and incandescent light, with a 14/10 hour light photoperiod. Temperature was 23 $\pm$ 1 °C in light and 16 $\pm$ 1 °C during the dark period.

Separation of cyanobionts

Plants were harvested at 14 days after inoculation, then washed in media that contained 25 mM of NaCl buffered with 10 mM of Tris-base and Tris-HCl, pH 6.0. *Azolla* plants were washed in the same media with 0.2% sodium hypochlorite + 0.01% Triton-X 100 for 20 min. Plants were rinsed 10x with the same media, and macerated by blender. Cyanobacterial cells were separated from plant debris by diluting the macerate in medium, filtering through 10 layers of cheesecloth, and pelleting by differential centrifugation. Pellets were resuspended in medium containing 2% pectinase, 2% cellulase and 0.05% of pectolyase (Sigma Chemical Co.), and incubated for 2 hr at 25 °C. Pellets were then washed 2x with medium, centrifuged at 500x g and fractionated by density gradient centrifugation in 10-90% Percoll at 2500x g (2). The cyanobacterial fraction was removed, examined microscopically for plant debris and for cellular integrity, and washed 2x in $BG11_o$ medium (10). Any residual plant debris was removed by repeating the gradient purification step. Cyanobacteria were then sonicated, centrifuged and filtered through 0.22 μm micropore prior to HPLC analysis.

Isolation of cavities

500g of *Azolla* plants were suspended in 500 ml of solution containing 1 % (w/v) cellulase, 1% pectinase and 0.01% of pectolyase (Sigma Chemical Co.). Flasks were incubated overnight with shaking at 80 rpm to separate cavities from the plants. Cavities were isolated by transferring the plant material in the enzyme solution to graduated cylinders and diluting to a liter with the same ionic-buffered solution less the enzymes. Floating material containing cavities was skimmed off and resuspended in 100 ml of fresh enzymatic solution and incubated for 2 additional hours. Floating cavities were further purified on a 10 to 30% Percoll gradient, washed in the medium, then gently washed over a 0.45 μm millipore filter, and quickly rinsed with deionized H_2O. Three g of freshly isolated cavities were expressed to obtain the internal sap which was then centrifuged and filtered through 0.22 μm micropore filter before analysis by HPLC. Internal air and liquid composition of the cavities was estimated using gravimetric and volumetric techniques.

Plant material

Cyanobacteria-free plants were ground with a mortar and pestle and the macerate centrifuged for 5 min. at 35,000g at 15 °C. Any contamination from cyanobacteria was assessed by scanning the supernatant for biliproteins by optical density between 500 and 700 nm.

Organic osmoregulatory compounds

Oligosaccharide content was determined using an Aminex 42A column (Bio-Rad), and simple sugars were determined with a Sugar Pak (Waters). The mobil phase was H_2O for the Aminex column, and 50 mg/l Na_2-CaEDTA for the Sugar pak, with flow rates of 0.5 ml/min. Columns were attached to a Waters HPLC system which was operated at 85 and 90 °C, respectively. Peaks were detected by refractive index (Waters model 401 Differential Refractometer) and identified by comparison with retention times of authentic standards.

RESULTS

The major difference in the oligosaccharide profiles of cyanobiont-free plants of *A. mexicana* and plants symbiotically associated with cyanobacteria was in the different degree of polymerizations (dps) in fraction 8 and 9 corresponding to retention times (rt) of 8.52 and 8.28, respectively (Figure 1). Cyanobacteria-free *Azolla* plants contained mainly dp 8 (rt 8.52) while the cyanobacteria association resulted in a predominance of dp 9 (rt 8.28). Area of peaks (data not shown) showed an almost complete equivalency. Figure 2 shows that the major oligosaccharides identified in Figure 1 were concentrated in *A. rubra* plants associated with cyanobionts and present in low concentrations in the cavity and in cyanobionts. This was in contrast to the amount of glycerol-like compound ("glycerol") found in the same components (see Table 1). "Glycerol" was present predominantly in cavities and in cyanobacteria.

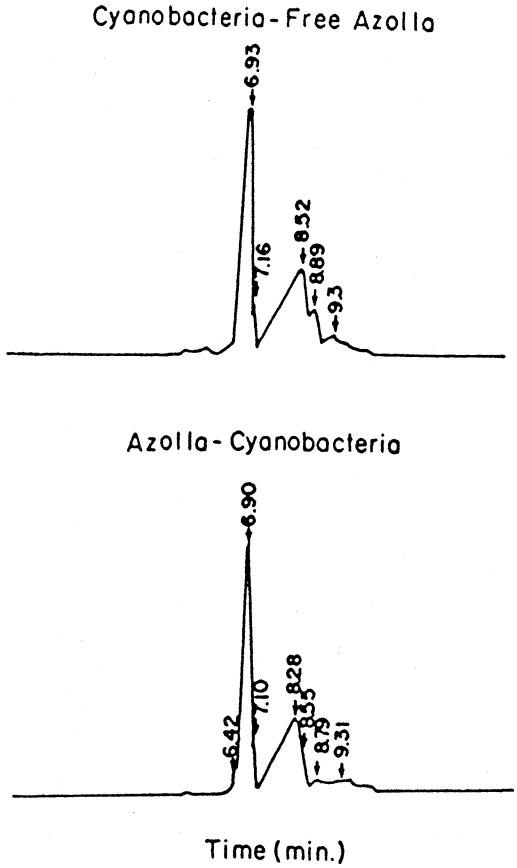

Figure 1. Oligosaccharide profiles of cyanobacteria-free whole plants of *Azolla mexicana* and plants symbiotically associated with cyanobacteria. Vertical axis is relative response. See text for conditions of elutions.

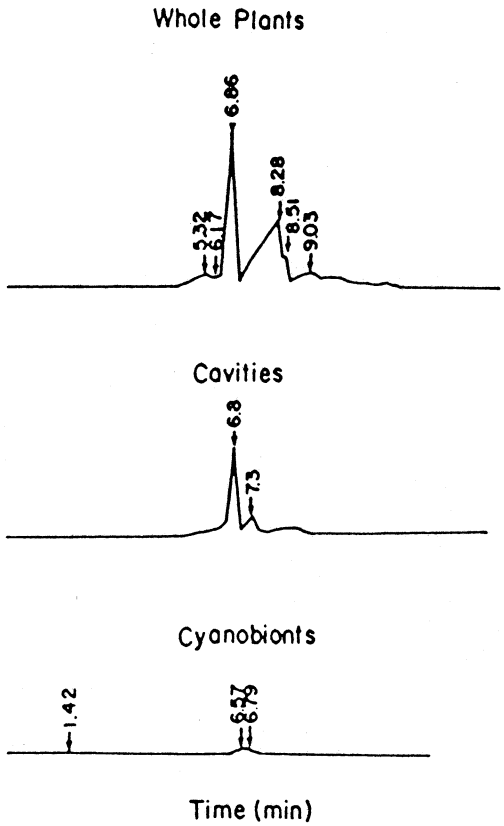

Figure 2. Oligosaccharide profiles of *Azolla rubra* compartments (whole plants, cavities and cyanobionts).

Sucrose was not found at detectable levels in the cavity nor in cyanobacteria, and glucose and fructose concentrations in the cavity were significantly higher than in the rest of the fern plant. "Glycerol" concentration in the cavity as well as in the cyanobacteria was dramatically higher than in the plant tissue. This substance could provide the osmotic potential in the cavity sap required by the cyanobacteria.

The ventral cavities of *Azolla* were composed of 90% of air and 10% of mucilaginous material.

Table 1. Concentration in mM of metabolites in cyanobacteria -free plants, in symbiotic *A. mexicana*, and in various components of the *A. rubra*-cyanobacteria association.

	A. mexicana		*A. rubra*		
Metabolites	AXF*	AX1	AR1	Cavities	Cyanobionts
Sucrose	0.16	0.78	0.17		
Glucose	1.83	5.64	2.68	35.50	1.70
Fructose	1.73	4.88	3.22	29.13	0.75
"Glycerol"	16.73	38.00	19.21	380.00	132.00

*. AXF- Cyanobacteria-free *A. mexicana*; AX1- *A. mexicana*, AR1-*A. rubra* whole plants.

DISCUSSION

A dynamic metabolic and osmoregulatory relationship appears to exist between *Azolla* and the symbiotic nitrogen- fixing cyanobacteria in the ventral cavities. The osmotic potential of the cavity can be assumed to be critical to the union, and is apparently maintained by a mixture of simple and complex sugars which, the data suggests, are derived after symbiosis.

Cyanobionts freshly extracted from cavities of *Azolla*, showed high concentrations of a compound that has been partly-identified as "glycerol" (with a similar retention time to a glycerol standard in HPLC), and minor concentrations of glucose and fructose. *Azolla* cavities (isolated by enzymatic digestion) contained high concentrations of the "glycerol", and significant amounts of complex-polymeric sugars, glucose and fructose. It may also be noted that monosaccharides (glucose and fructose) rather than sucrose are accumulated in the cavity as fixed carbon.

Sucrose was not found in cyanobionts nor in cavities, which is in partial agreement with the data of Tel-Or et al. (11). Cyanobacteria-free *Azolla* plants had "glycerol", and had only low levels of complex and simple sugars. The cavity concentration of "glycerol" was high in comparison to the rest of the plant, implying that it is mainly located in the cavities of *Azolla*. This compound may provide suitable reserve energy and an osmotic environment for the cyanobionts. "Glycerol" concentrations in cavities would contribute nearly -10 bars of osmotic pressure (data extrapolated from tables of Harris, (1981). Inorganic ions contribute -3 bars of pressure (1). Apparently high osmolarity is needed in cavities for proper cyanobacterial growth and nitrogen fixation.

This study also reveals that the association of the *Azolla* fern and the cyanobacteria results in a change in the oligosaccharide content of whole plant extracts. The data implies that cyanobionts induce the build-up of carbohydrates needed as an energy source for nitrogen-fixation.

We are currently extending these studies to determine whether enzymes of carbohydrate metabolism are induced in the cavity area by the cyanobacteria. Also, we are using various techniques which include NMR and FAB-MS (fast atom bombardment-mass spectrometry) in determining the identity of "glycerol" and the components of the oligosaccharide mixture.

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