# Studies on the Photosynthetic Carbon Acquisition of Azolla-Anabaena Symbiosis

**Mie Mie Aung[1], Ulrich Eberhardt[2], Paul L. G. Vlek[3]**

[1]Georg-August University Göttingen, Institute of Agronomy and Animal Production in the Tropics and Subtropics, Germany
[2]Georg-August University Göttingen, Former Central Isotope Laboratory, Germany
[3]University of Bonn, Center for Development Research, Germany

## Abstract

*Azolla spp.* is small ferns floating on water surfaces. They contain cyanobacterial microsymbionts (*Anabaena azollae*) in leaf cavity which are able to fix elemental nitrogen. The combination of photosynthetic acquisition of carbon and energy and biological nitrogen fixation provides a basis for potentially high productivity of the symbiotic system. Consequently, Azolla have been used for centuries to fertilise rice paddies without need for supplementary manure. When combined with urea as a fertilizer, Azolla layers reduce the volatilization of ammonia originating from urea hydrolysis by decreasing the pH of floodwater. This effect is accomplished by reduction of algal photosynthetic activity during the day. More insight into the physiology of Azolla system and into the interactions between Azolla and Anabaena may increase the benefit from using Azolla in agriculture and in this way contribute to a sustainable land use. Photosynthesis is the ultimate source of ATP and reductant required for nitrogen fixation and there is a close relationship between both processes. Thus carbon fluxes into and out of Azolla and between the symbiotic partners are of interest. Studying *Azolla caroliniana* and *A. pinnata* var. *imbricata* involving the measurement of $^{14}C$ incorporated from $^{14}CO_2$ into symbiotic Anabaena filaments and Azolla tissues, we found that less than 15% of the fixed carbon in the system was located in the Anabaena. Kaplan and Peters (1988), comparing net photosynthesis rates of symbiotic association and Anabaena-free Azolla plants, suggested that Anabaena contributes little to carbon fixation. Sucrose is assumed to be the photosynthetic product transferred from Azolla to Anabaena (Kaplan & Peters, l.c.). Some approaches to verify the existence of carbon metabolism in Anabaena symbionts are presented.

**Keywords:** Azolla, carbon acquisition

**Contact Address:** Mie Mie Aung, Georg-August University Göttingen, Institute of Agronomy and Animal Production in the Tropics and Subtropics, Grisebachstraße 6, 37077 Göttingen, Germany, e-mail: maung@gwdg.de

## 1. Introduction

Azolla, floating on the water surface, is a genus of aquatic, heterosporous ferns that normally contains a symbiotic, heterocystous cyanobacterium, *Anabaena azollae*, within cavities formed in their aerial dorsal leaf lobes.. Under optimised conditions these associations can double their biomass in less than two days with dinitrogen as the only N source and accumulate 5-6.5% N based on a dry weight (Peters et al., 1980). Azolla-Anabaena symbiosis is noted for its high productivity combined with its ability to fix dinitrogen at high rates. Consequently Azolla has been used for centuries to fertilize rice paddy avoiding the need for manure.

Both partners of the association are photosynthetically competent and their pigments are complementary. However, there are clear indications that the microsymbiont contributes only little to the total carbon fixation of the Azolla-Anabaena symbiosis and that carbon required by the endophyte to a large part is provided by the host (Kaplan & Peters, 1988). ATP necessary to drive nitrogen-fixation seems to be produced photosynthetically in the Anabaena-heterocysts. We present some experiments to elucidate the participation of Anabaena in the C-assimilation of the symbiosis.

## 2.Materials and Methods

Plant material and growth conditions

*Azolla carolinia* and *Azolla pinnata* var. *imbricata* were grown on N-free medium. For Anabaena-free Azolla plants, 4 mM potassium nitrate was added to the medium. All cultures were maintained using a cycle of 15 hr light (28°C) and 9 hr dark (18°C). HQI-T lamps (400 W) provided a light intensity of 110 µE $m^{-2}s^{-1}$.

$CO_2$ exchange rates under light and dark

Whole fronds of the association and the endophyte-free plants were harvested and weighed. Samples were placed on 1 L of growth medium in 5 L glass vessel which were aerated by an air stream of 15 L/h under fluorescent light of 45 µE $m^{-2}s^{-1}$. The experimental light and dark phases were 14 and 10 h, respectively. Rates of $CO_2$ uptake and release under light (14 h) and dark (10 h) were determined from $CO_2$-concentrations in the air stream into and out of the reaction vessel, measured By gaschromatograph.

$^{14}CO_2$ fixation of Azolla and participation of Anabaena

$^{14}CO_2$ experiments with the symbiotic association and Anabaena-free *Azolla caroliniana* were performed using 5 L glass vessel containing 1L of respective growth medium. A weighted plant sample was placed on the medium surface and allowed to equilibrate at 27°C and 45 µE $m^{-2}s^{-1}$ for 60 min in normal air. Then, the reaction vessel was sealed with the lid. In the course of 4 h, a solution of $Na_2{}^{14}CO_3$ containing 1850 kBq of $^{14}CO_2$ was gradually injected. After the final carbon dioxide concentration in the atmosphere was decreased to 200ppm, the lid of the vessel was removed. As rapidly as possible some fronds were weighed and dropped into liquid nitrogen in near darkness to measure the total $^{14}C$ content in biomass. Other fronds known weight were used for isolation of the endophyte.

For isolation of the endophytic Anabaena, fronds were suspended in the nitrogen-free medium and ground for 30-60 sec in a motor driven, Teflon homogenizer. The homogenized materials were sequentially passed through 1 layer of tea filter paper and a 110 µ nylon mesh, followed by rinsing the residue with the medium, to separate Anabaena filament from plant debris. The filtrates were centrifuged at 1600 g speed for 17 min and then the supernatant decanted. A pellet, containing Anabaena filaments free from plant debris was designated filament fraction for use in specific studies. The materials remaining on the filter paper, containing both plant debris and the rest of Anabaena filaments, was resuspended in the

growth medium, centrifuged as before, the supernatant decanted and the pellet was designated residual fraction.

Radioactivity was measured by liquid scintillation counting after incinerating the material in an automatic device to form $^{14}CO_2$.

While the endophyte could be isolated free of the fern, its removal from the host was never complete. Thus it was necessary to find an independent quantitative measure for the amount $^{14}C$ content in Anabaena filaments. To estimate the relative amount of Anabaena filaments in the two fractions, the $N_2$ fixation rate of filament fraction and the residual fraction was measured by the acetylene reduction assay (ARA) method (Tung and Shen, 1981). The assumption is that nitrogenase is unique to the Anabaena (Peters and Mayne, 1974),

## 3. Results and discussions

The rate of fixation by the association and endophyte-free plants were determined in the atmospheric air. As shown in Table 1., the rate of $CO_2$ exchange and all subsequent results are expressed on the basis of dry matter. No important difference in $CO_2$ assimilation rates between the Azolla-Anabaena-system and Anabaena-free ferns were found. Enhanced respiration rates of Anabaena-free plants were noted in the dark.

Table 1. Photosynthetic capacity of the Azolla-Anabaena symbiosis and Anabaena-free Azolla

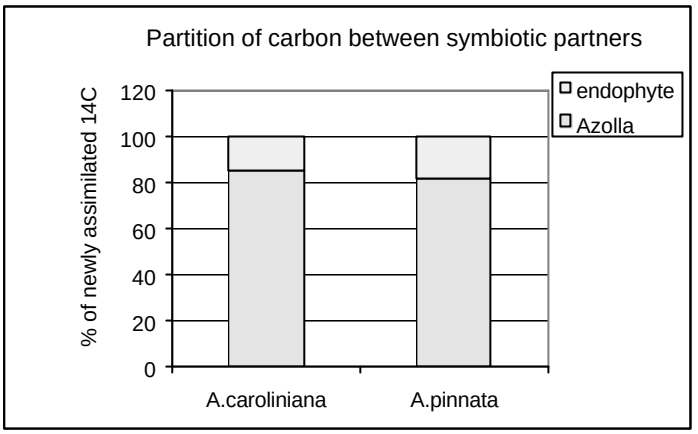
| Parameter | Azolla pinnata | Azolla caroliniana | | |
|---|---|---|---|---|
| | | Symbiosis | Anabaena-free | Difference (% of) from symbiosis |
| $CO_2$ fixation rate (μ mole $CO_2$ / g dm. h) | 231 | 204 | 195 | 0 |
| $CO_2$ releasing rate (μ mole $CO_2$ / g dm. h) | 96 | 68 | 138 | +100 |
| Net C yield (for light-dark cycle) (μ mole $CO_2$ / g dm.) | 2260 | 2160 | 1350 | -38 |

In Anabaena-free plants in the presence of nitrate there is no necessity to maintain the cyanobacterial population and to perform energy-consuming nitrogen fixation. However, nitrate has to be reduced to ammonium and this may compensate to a large part for the saving in carbon. The high respiratory carbon loss and the lower net C-yield may be one cause to explain the very slow growth of Anabaena-free Azolla. It may, however, be that the Anabaena-free Azolla is a system not comparable to Azolla-Anabaena symbiosis based on the differences in $CO_2$ fixation rate of Azolla and Anabaena-free Azolla. The association and Anabaena-free Azolla both fix $CO_2$ by the Calvin cycle and have sucrose as the major fixation product. The endophyte also fixes $CO_2$ via the Calvin cycle when removed from the fern.

We employed a long term experiment with $^{14}CO_2$ to assess the amount of C transported from Azolla to its endophyte. The total $^{14}C$ of the association and its components, the filament and residual fraction were measured. The ARA of both fractions was determined and these were taken together as that of the total Anabaena-population. The $^{14}C$ content of the Anabaena-population was calculated from $^{14}C$ content in the filament fraction, corrected for its share in total ARA.

The total label in the endophyte accounted for less than 15% of that in the association after long term labelling. Under these controlled conditions the endophyte isolated from *A. pinnata* has a slightly larger amount of total $^{14}C$ compared to that from *A. caroliniana*. These data were interpreted to suggest that Azolla provide about 15 % of its fixed carbon to the

endophyte. In rapidly growing laboratory cultures of the association, *A. azollae* accounts for less than 20% of the association's chlorophyll and about 16% of the total protein (Ray, et al., 1978). While the endophyte is capable of fixing $CO_2$ as part of the symbiosis, its contribution to the total $CO_2$ fixation of the association under its optimal growth conditions is less than 5% and Anabaena, the endophyte, in the leaf cavities is mostly dependent on the Azolla for its carbon compounds (Kaplan & Peters, 1988).

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