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# PHOTOSYNTHESIS AND $N_2$ FIXATION IN THE AZOLLA-ANABAENA SYMBIOSIS

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*Azolla* is a genus of aquatic ferns which contains an $N_2$-fixing cyanobacterium, *Anabaena azollae*, as an endophyte. Under optimal growth conditions these associations double their biomass within two days, and contain 5-6% N on a dry weight basis, with $N_2$ as the only N source (6). Using the optimal growth temperature for the individual species (6), comparative studies of growth, photosynthesis, $N_2$ fixation, and related processes have revealed no major differences in four of the six known *Azolla* species. Only *A. caroliniana* is considered here.

## DEVELOPMENTAL MORPHOLOGY AND PHYSIOLOGY

The apical region of the fern contains undifferentiated *Anabaena* filaments which do not fix $N_2$. During leaf cavity development the endophyte rapidly differentiates heterocysts and this is paralleled by increasing nitrogenase activity (3,5). Epidermal hairs which line the leaf cavities (7) are comprised of two types: simple and branched (1). While the number of simple hairs increases roughly in parallel with nitrogenase activity, with 20 to 25 in each mature cavity, there are only two branched hairs per cavity. Moreover, while the simple hairs are randomly distributed, the branched hairs are always located along the path of the foliar trace. Related studies have shown that the N content and dry matter are highest in the apical region with the C/N ratio increasing as a function of leaf age and that $N_2$ fixed in mature leaves is transported toward the apical region. It is postulated that the hairs function in the transfer of metabolites between the partners and that the endophyte exhibits mixotrophic metabolism, undergoing a transition from photoautotrophic to photoheterotrophic metabolism as a function of leaf age.

## PHOTOSYNTHESIS AND $N_2$ FIXATION

The *Azolla-Anabaena* association is comprised of two photosynthetically competent organisms and the endophyte's phycobilins (13) complement the fern pigments in absorbing solar radiation (8). Although the endophyte accounts for about 20% of the association's chlorophyll and protein (9), its contribution to the total photosynthetic capability of the association has not been resolved. The association has the characteristics of a C-3 plant: an $O_2$ inhibition of photosynthesis (PS), an $O_2$-dependent $CO_2$ compensation point and Calvin cycle intermediates of $CO_2$ fixation with sucrose as the primary end product (8).

All of the ATP and reductant required for nitrogen fixation is ultimately derived from PS. Dark, aerobic $N_2$ fixation is dependent upon endogenous reserves of photosynthate. Its rates are never more than half of those obtained aerobically in saturating light and may be ATP limited (4,5). Photosynthetically-driven nitrogen fixation is likewise dependent upon photosyntnate but inhibition of Photosystem II (PSII) has little effect on nitrogenase activity until endogenous reserves of carbon compounds are depleted. This indirect role of PSII and non-cyclic photophosphorylation implicates cyclic photophosphorylation associated with Photosystem I (PSI) as the primary source of ATP for photosynthetically-mediated $N_2$ fixation and is consistent with nitrogenase being localized in heterocysts.

The action spectra for PS in the association and isolated endophyte are similar to those of other green plants and cyanobacteria, respectively, and both exhibit the characteristic red drop, i.e., low quantum yield in the region of chlorophyll a absorption (8). In contrast, the action spectra for nitrogenase activity ($C_2H_2$ reduction) in the association and endophyte do not exhibit this red drop and are consistent with a PSI-linked process. However, the relative quantum yields for $C_2H_2$ reduction activity (ARA) in the region of phycobilin absorption equal or exceed those in the region of chlorophyll a absorption (12). Phycobilins are generally associated with PSII of vegetative cells (2) and were found to be the primary pigments capturing light energy for PS in the endophyte (8). However these accessory pigments, which absorb light energy in the region of minimal absorption by the plant tissue, also capture solar energy for the PSI-linked process of $N_2$ fixation.

## EFFECT OF COMBINED N SOURCES

In an initial study of the effect of combined N sources on growth, PS and $N_2$ fixation, growth rates of A. caroliniana were generally comparable using 0 to 5 mM ammonium, 0 to 25 mM nitrate or 0 to 12.5 mM urea, with doubling times between 1.9 and 2.4 days (4). After 3 to 5 weeks growth on each concentration of combined N, $^{15}$N-labeled combined N was used to determine absorption rates and isotope dilution was used to determine the input from the combined N source versus $N_2$ fixation. For example, these studies showed that with 2.5 mM ammonia, 10 mM nitrate and 5 mM urea, 64%, 77% and 56%, respectively, of the N input was from $N_2$ fixation. In contrast to the effect of ammonia or urea, $H_2$ production did not decrease in parallel with ARA with growth on increasing concentrations of nitrate (4).

## PHOTOPERIOD, PHOTOSYNTHESIS AND $N_2$ FIXATION

Previous studies showed that the growth rate of four *Azolla* species increased with increasing day length (6). A study of the effect of varying light regimes (12 hr, 16 hr and continuous light) on physiological processes in A. *caroliniana* revealed few statistically significant differences in rates of PS, respiration, nitrogenase-catalyzed reactions and related processes between plants grown under 12 hr-12 hr and 16 hr-8 hr light dark regimes (4). Table 1 provides an abbreviated presentation of similar data. In conjunction with these studies, aerobic ARA and $^{15}N_2$ fixation were determined at the midpoint of the light and dark intervals of the two photoperiods. The values for $C_2H_2$ reduced/$N_2$ fixed fell between 4.07 and 4.40 and it was estimated that light, aerobic $N_2$ fixation contributes 73% of the total daily N during a 12 hr light period and 81% during a 16 hr light period (4). As shown in Table 1, nitrogenase-catalyzed $H_2$ production under Ar-$O_2$-$CO_2$ is always much less than aerobic ARA in the light and $H_2$ production under air is negligible. This is consistent with the occurrence of an uptake hydrogenase and a high relative efficiency of $N_2$ fixation in these associations.

In attempting to relate measurements of physiological processes such as PS, respiration and $N_2$ fixation to the observed growth rates and %C and %N of the tissue a number of factors come into play. Among others, these include the inherent biological variability of plants within a population and the fact that whereas growth, %C and %N represents cumulative responses over days or weeks, measurements of physiological processes are usually restricted to short term assays. Figure 1a and b shows the daily course of PS and ARA in the light and respiration and ARA in the dark with A. *caroliniana* grown under 16 hr-8 hr (a) and 12 hr-12 hr (b) light-dark regimes. Although the light and dark determinations were made on separate cultures they are considered comparable. By integration of the area under the respective curves, subtracting respiration from PS and adding light and dark ARA with conversion to $N_2$ (using conversion factors determined with $^{15}N_2$; 2.15 and 4.09 $C_2H_2/N_2$ for 12 hr and 16 hr light period, 3.28 and 4.36 for 12 hr and 8 hr dark periods, respectively) and knowing the %C and %N, it is possible to estimate doubling times on the basis of daily C and N input. These values can then be compared with the observed doubling times based on weight. In so doing we obtain a doubling time of 2.8 days based on carbon input, 2.5 days based on N input and an observed value of 2.7 days for the 12 hr-12 hr regime. For the 16 hr-8 hr regime the

Table 1. Photosynthesis, $N_2$ Fixation and Related Processes in A. *caroliniana* Grown under two Photoperiods

| | 12 hr - 12 hr | 16 hr - 8 hr |
|---|---|---|
| CER, air-light | 123.05 ± 9.5 | 117.20 ± 6.2 |
| CER, air-dark | 15.69 ± 2.8 | 14.30 ± 1.2 |
| $CO_2$ compensation point | 40.00 ± 0.3 | 44.00 ± 2.1 |
| ARA, air-light[a] | 1.83 ± 0.3 | 1.61 ± 0.4 |
| ARA, air-light[b] | 2.58 ± 0.2 | 2.86 ± 0.2 |
| ARA, air-dark[a] | 0.86 ± 0.1 | 0.67 ± 0.1 |
| HPA, Ar-$O_2$, light[b] | 0.18 ± 0.0 | 0.46 ± 0.0 |
| HPA, air-light[b] | 0.01 ± 0.0 | 0.00 ± 0.0 |
| Percent dry matter | 5.35 ± 0.5 | --- |
| Percent C | 43.60 ± 0.9 | --- |
| Percent N | 5.71 ± 0.4 | --- |

CER = $CO_2$ exchange rate, ARA = acetylene reduction activity, HPA = hydrogen production activity. Values expressed as μmoles production/g fr wt-hr except for compensation point which is ppm $CO_2$. Growth conditions: IRRI medium buffered at pH 6 with 10 mM MES, constant temperature of 25°C, light intensity of 300 μE/$m^2$·sec (6). Assayed under growth conditions. [a]Determined simultaneously with CER; [b]Determined in vials.

respective values are 2.0, 3.5 and 2.2 days. (A doubling time of 2.4 days instead of 3.5 is obtained here if a theoretical conversion factor of 3.0 $C_2H_2/N_2$ is employed.) In comparison to the estimates of the contribution of light, aerobic $N_2$ fixation to daily N input noted above, the integrated values for ARA in Figure 1 represent 85% of the total input during the 12 hr light, 90% during the 16 hr light period.

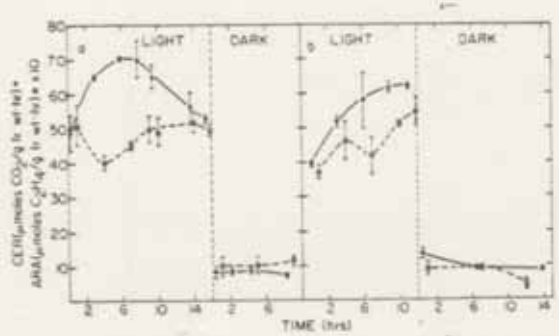


Figure 1. Apparent photosynthesis and acetylene reduction activity in the light and respiration and acetylene reduction in the dark during the 24 hr cycle of: (a) 16 hr-8 hr and (b) 12 hr-12 hr light-dark regimes.

SPECULATION ON THE CARBON COST OF $N_2$ FIXATION

The carbon cost of $N_2$ fixation in actinorhizal and legume nodules, approximated by the ratio of respiration to nitrogenase activity, has yielded values for $CO_2/C_2H_2$ ranging from 3.4 to 5.6 for legume and 2.8 to 8.7 for actinorhizal nodules (11). In the *Azolla*-*Anabaena* symbiosis, photosynthetically-mediated ARA is usually two to four times greater than the maximal dark-aerobic ARA. If one assumes that dark activity is ATP-limited and that the rate of respiration measured in the dark during the light period is equivalent to that in the light, it is possible to estimate the umoles $CO_2$ respired/$C_2H_2$ reduced during photosynthetically-mediated $C_2H_2$ reduction. In the *A. caroliniana* association, a total of 8 determinations from plants grown under 12 hr or 16 hr light regimes yielded values of 17.11 ± 4.76 umoles $CO_2$ respired/g fr wt·hr and 2.10 ± 0.57 umoles $C_2H_2$ reduced/g fr wt·hr for a $CO_2/C_2H_2$ ratio of 8.15. If one further assumes that the partners contribute equally to the association's respiration on the basis of chlorophyll or protein, the endophyte's contribution would be about 20% of the total (9). This would equate to 3.42 ± 0.95 umoles $CO_2$ respired/g fr wt·hr and 2.10 ± 0.57 umoles $C_2H_2$ reduced with a $CO_2/C_2H_2$ ratio of 1.63. Employing a $C_2H_2/N_2$ conversion factor of 4.0, this equates to 6.5 moles C/mole $N_2$ or 2.8 mg C/mg N.

Although it is possible to isolate the endophyte, previous studies indicated that nitrogenase activity in the isolate was 30 to 50% less than its estimated activity in the association (9) and that, in the association, the endophyte may be provided with fixed carbon from the fern. Thus, no attempt has been made to determine the $CO_2/C_2H_2$ ratio in the isolated endophyte. Furthermore, we have no evidence that in the light, as in the dark, it is respiration rather than PS which supplies the reductant for nitrogen fixation.

In comparison to the above, utilizing the dark respiration and dark ARA during the light period (data not shown), assuming that the endophyte accounts for 17% of the association's respiration and a $C_2H_2/N_2$ value of 3.55 (based on the mean from several determinations in the light and dark with 12 hr, 16 hr and continuous light periods for each of four *Azolla* species), the mg C/mg N for the four *Azolla* species under 12 hr and 16 hr light periods ranged from 2.95 to 7.03 with a mean of 3.14 ± 1.45 (O. Ito, personal communication). For *A. caroliniana* the mg C/mg N for dark fixation was 3.47 for the 12 hr-12 hr photoperiod and 4.62 for the 16 hr-8 hr. The higher C/N cost in the dark than in the light, using the same basic assumptions, is of course the result of the lower ARA in the dark.

Another approach to assessing the carbon cost involves the use of *Azolla* with and without the endophyte in a rough analogy to nodulated and non-nodulated legumes. Two points should be noted. First, the endophyte-free plants always grow more slowly than the association and it is possible that the absence of the endophyte creates a hormonal imbalance.

Second, the associations are able to utilize combined N and $N_2$ simultaneously (4). Using the data in Table 2, growth rate constants were determined and used to calculate the daily input of C and N required to maintain the C and N content of the plant material. These values were 5.6 mg C and 0.55 mg N for the endophyte-free *Azolla*, 9.21 mg C and 1.28 mg N for the association grown on $N_2$, and 9.55 mg C, 1.33 mg N for the association provided with ammonia as well as $N_2$. In comparing the endophyte-free *Azolla* with the association grown on $N_2$, the carbon requirement for $N_2$ fixation was estimated by (5.6 - 9.2) mg C/1.28 mg N = -2.81. In comparing the association provided with ammonia and the association grown only on $N_2$ one obtains (9.55 - 9.21) mg C/1.28 mg N = 0.27 mg C/mg N. [In the association provided with 2.5 mM $NH_4^+$ approximately 35% of the N input is derived from $NH_4^+$ (4)].

Table 2. Information Concerning *Azolla* with and without the Endophyte Used in Estimating Carbon Requirement of $N_2$ Fixation[1]

| | N Source | Doubling Time (days) | % Dry Matter | %C | %N |
|---|---|---|---|---|---|
| *Anabaena*-free *Azolla* | 2.5 mM $NH_4^+$ | 3.38 ± .25 | 6.00 ± .10 | 41.2 ± .10 | 4.03 ± .08 |
| *Azolla* with *Anabaena* | $N_2$ | 1.97 ± .10 | 5.22 ± .06 | 41.8 ± .03 | 5.54 ± .19 |
| *Azolla* with *Anabaena* | 2.5 mM $NH_4^+$ and $N_2$ | 1.89 ± .02 | 5.17 ± .14 | 41.7 ± .16 | 5.81 ± .10 |

[1]Plants grown as described in Table 1. Percent dry matter, %C and %N as described in (6).

In contrast to the mg C/mg N values calculated using respiration and $C_2H_2/N_2$, the comparison between the association grown on $N_2$ and either the endophyte-free plants or the association provided with combined N implies that the carbon cost to these photosynthetic associations is no greater for $N_2$ fixation than it is for ammonium uptake and metabolism. It is acknowledged that the above approximations of the carbon cost are overly simplified and purely speculative. It is also noted, however, that whereas the estimates from respiration and $C_2H_2$ reduction are within the 1-10 mg C/mg N range reported for legumes and actinorhizal associations (10,11), this ratio reflects the carbon respired for growth, maintenance, synthesis and transport phenomena as well as for nitrogenase activity. We have determined maintenance respiration values and it is apparent that this and other determinations must be made to obtain an unequivocable result with respect to the carbon cost of $N_2$ fixation in *Azolla*.

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