Distribution of the N_2 Fixation and Photosynthetic Activities in the *Azolla-Anabaena* Symbiosis

SABHARATHNA A. KULASOORIYA[1], HANNA ARAD[2], ORA CANAANI[3], ELISHA TEL-OR[2] and SHMUEL MALKIN[3]

[1] *Department of Botany, University of Peradeniya and Institute of Fundamental Studies, Kandy, Sri Lanka*
[2] *Department of Agricultural Botany, Hebrew University of Jerusalem, Rehovot, Israel*
[3] *Department of Biochemistry, Weizmann Institute of Science, Rehovot, Israel*

Received May 18, 1988; Accepted July 28, 1988

Abstract

Photosynthetic and nitrogenase activities of *Azolla filiculoides*, *A. caroliniana* and *A. pinnata* var. *imbricata*, were monitored by the photoacoustic and acetylene reduction techniques respectively, comparing different segments of the *Azolla* fronds. Both activities were very low in the apical parts, indicating that these processes are still not fully developed in them. The photosynthetic oxygen evolution quantum yield and photochemical energy storage were significantly higher in the middle segments compared to the lower segments. In contrast, specific N_2 fixation activity was much higher in the lower segments than in the middle segments, while the heterocysts abundance was similar. These results demonstrate an unequal distribution of the two major metabolic processes along the stem axis of the *Azolla* plant, and are consistent with the idea that part of the photosynthates synthesized in the middle segments may be translocated to support the high N_2 fixing activity in the lower segment.

Keywords: photosynthesis, N_2 fixation, photoacoustics, *Azolla-Anabaena*

1. Introduction

In the *Azolla-Anabaena* symbiosis, the *Azolla* fronds synthesize and provide photosynthates essential for the nitrogenase activity of the cyanobiont *Anabaena azollae* which is accommodated in the leaf cavities of the host (Peters et al., 1985). The two partners in the symbiosis undergo a mutual developmental process characterized by an increase of photosynthetic activity and a rapid growth of the host's fronds, concomitant with a vegetative proliferation and heterocyst differentiation in the cyanobiont. Hill (1975, 1977) studied acetylene reduction and heterocyst differentiation of *Anabaena azollae* in the leaves along the frond stem axis of *Azolla*. The distribution of nitrogenase activity along the main stem axes of *Azolla* was followed in *Azolla caroliniana* (Kaplan and Peters, 1981) *Azolla pinnata* var. *pinnata* (Becking and Donze, 1981) and *Azolla pinnata* (Kaplan et al., 1986). Nitrogenase activity was located mainly between leaves 8–20 in *Azolla filiculoides*, 3–12 in *Azolla caroliniana*, 2–10 in *Azolla pinnata*, and in two separate loci between leaves 4–17 in *Azolla pinnata* var. *pinnata*. The lower part of the stem was therefore assumed to be relatively inactive in terms of N_2 fixation. We found it interesting to determine whether photosynthetic activity parallels that of N_2 fixation. Such information seemingly does not exist, due to the lack of proper methodology for the analysis of gross photosynthesis *in situ*.

The photoacoustic technique provides a rapid and reliable tool to measure *in vivo* the quantum yields of O_2 evolution and energy storage in the photochemical reactions of photosynthesis (Bults et al., 1982; Poulet et al., 1983; Buschman et al., 1984). It involves the detection of modulated heat emission and modulated gas exchange from an intact leaf or a lichen resulting from the absorption of intensity modulated light. Acoustic signals arise following propagation of the modulated heat and oxygen, which reach the cell boundary and generate periodic pressure modulation (i.e. sound) in the adjacent air layer. The sound is then detected by a microphone and recorded as a "photoacoustic signal". Photoacoustic signals resulting similarly from modulation in CO_2 uptake are not expected to play a significant role because they are presumably completely damped in the range of frequencies between 10–100 Hz used in our experiments (Bults et al., 1982). Since photoacoustics involves a modulation technique, it is related to *gross* photosynthesis at light limiting conditions, and is insensitive to respiration and presumably as well to photorespiration.

In this report, we show results of photosynthetic activities measured in the symbiotic system *Azolla-Anabaena* by the photoacoustic method. The

distribution of these activities in defined segments along the stem axes of *Azolla* fronds were correlated with the activity of N_2 fixation carried out by the cyanobiont. Interestingly, we found an unexpected converse relationship between photosynthesis and N_2 fixation in the middle and lower segments of the *Azolla* fronds, which was confirmed in three different *Azolla* species.

2. Materials and Methods

Plant material

Azolla filiculoides, *Azolla caroliniana* and *Azolla pinnata* var. *imbricata* were grown in the phytotron of the Department of Agricultural Botany at temperatures of 27°C/22° C under a light/dark regime of 16:8 hr, respectively. *A. filiculoides* was grown in Hoagland medium diluted 1:8, *A. caroliniana* and *A. pinnata* var. *imbricata* were grown in the International Rice Research Institute (IRRI) medium (Watanabe et al., 1977).

Acetylene Reduction

Plantlets or segments were floated in 12 ml serum bottles containing 2 ml growth medium, 10% acetylene in air and sealed with rubber stoppers. Samples were illuminated (light intensity 5 Wm^{-2}) on a shaker at 25°, for 1 hr. Ethylene production was monitored by a Gow-Mac model 69–100 gas chromatograph provided with a Poropack N column and flame ionization detector.

Cell counts

Representative leaves from each segment were crushed in water, on a Haemocytometer counting cell, and the heterocysts and the vegetative cells of *Anabaena azollae* were counted under a phase contrast microscope, using a digital counter. Heterocyst frequency was calculated on a percentage basis, from mean values of three counts, counting at least 400 cells each time.

Photoacoustic apparatus

The photoacoustic technique and apparatus has been previously described in detail (Bults et al., 1982; Poulet et al., 1983). A quartz-iodine (250 W) lamp was used as the exciting light source and was modulated by the use of a mechanical chopper (Laser Precision). Narrow-band monochromatic light was isolated with the use of 2-cavity interference filters (Ditric Optics). Non-modulated saturating background light of spectral band between about 400–680 nm, isolated with a short-pass interference filter (Ditric Optics), was obtained from a d.c. operated quartz-iodine 250 W lamp. The modulated and

background lights were passed to the *Azolla* plants through fiber optic light guides. The acoustic signal was detected by a microphone (Knowles) and analyzed after filtered preamplification by a lock-in amplifier (Brookdeal). Light intensities were measured with a calibrated radiometer (Yellow Springs Instruments).

Procedures and analysis of the photoacoustic signals

Representative photoacoustic data are shown in Fig. 1. Most of the measurements were carried out either at 19–22 Hz ("low frequency") or at 400 Hz ("high" frequency). At the high modulation frequency the O_2 evolution oscillations are completely or largely damped and only the heat contribution (photothermal signal) remains. Under these conditions the addition of non-modulated light of saturating intensity, which by itself cannot create any microphone signal, drives the system to photosynthetic saturation, thereby closely approaching the situation of full conversion of light to heat. This is reflected by an increase in the microphone signal. The fractional increase, relative to the achieved maximum signal, is termed "photochemical loss" (P.L.), and is a measure of energy storage due to the photochemical conversion of light energy, hence a measure of the efficiency of photosynthesis. At low modulation frequency, O_2 evolution contributes to a large extent to the signal, as it is superimposed with the photothermal component. Addition of saturating background light eliminates the modulated light-induced oscillations in O_2 evolution (since the rate at saturation is light-intensity independent), resulting in an appreciable decrease of the signal to a value reflecting the maximum photothermal conversion alone. The O_2 signal is then routinely obtained by subtracting the photothermal signal from the total photoacoustic signal, usually after correcting the maximum photothermal signal by the P.L.* One has to consider that the phases of the photothermal and O_2 evolution modulations are not equal, hence one has to treat the signals as (mathematical) vectors, by their in-phase (I) and quadrature (Q) components (Poulet et al., 1983), as monitored by the lock-in amplifier. Routinely (but not necessarily), as shown in Fig. 1, when the background light was on, the phase

* One should consider the possible objection that the P.L. was measured at the "high" frequency while the P.L. correction that should be applied is that appropriate to the applied frequency. The effect of this inaccuracy, which is at present unavoidable, is relatively small. Even without any such correction the values of the uncorrected oxygen signal and P.L. change roughly in a parallel manner. This gives a very similar quantitative trend when comparing corrected and uncorrected O/T values.

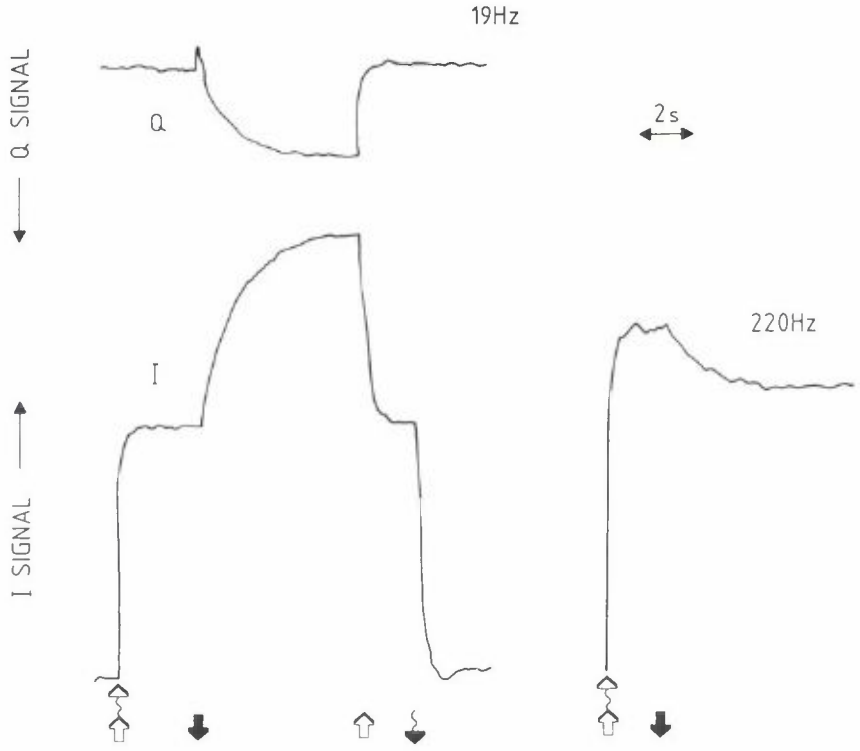

Figure 1. Example of raw data: Photoacoustic imphae(I) and quadrature (Q) signals from *Azolla pinnata* segments, measured at two modulation frequencies: "low" (20 Hz) and "high" (400 Hz). The arrows in the I and Q axes indicate the direction of the signal increase. Modulated light, 680 nm, 14 W/m^2. Saturating background light 400–680 nm, 360 W/m^2. ⇧ modulated light on, ⇩ off, background light on, ↓ off.

The PL is calculated from the high frequency data as: (signal with background light — signal without background light) / (signal with background light). The oxygen signal (O) is calculated from the low frequency data as: $\sqrt{O_I^2 + O_Q^2}$, where $O_I(O_Q)$ are respectively the I (or Q) signal without background light minus I (or Q) signal with background light × (1 – PL).

The maximal photothermal signal (T) is the I signal in presence of background light. From the above data O/T may be calculated.

control on the lock-in amplifier was adjusted so that the quadrature was zeroed and hence the photothermal signal appeared as an in-phase component only. When the background light was switched off, the in-phase signal increased and was a sum of O_2 and photothermal in-phase components while

the quadrature signal increased from zero and was due to O_2 evolution soley. the total signal is thus the *vectorial* sum of the O_2 evolution and photothermal vectors. The amplitude of each vector is calculated by the square root of the sum of squares of the vectorial components. Since the amplitude of the O_2 evolution vector, O,is proportional to the absorbed quantal flux times the quantum yield and that of the photothermal vector, T, is proportional to the same flux times the photon energy, the ratio O/T is directly proportional to the relative quantum yield of O_2 evolution.

The photoacoustic measurements for a single sample held inside the apparatus were very reproducible and no essential differences were noticed after an hour in continuous monitoring. Any individual determination actually takes a few seconds.

3. Results and Discussion

One of the aims of this investigation was to establish a rapid and practical method of a combined analysis of the productivity of *Azolla*, with an initial attempt to compare nitrogenase activity and photosynthetic activity along three different loci along the stem axes of the *Azolla* fronds.

The segments chosen were: the apex, the middle and the lower segment, shown for *Azolla filiculoides* in Fig. 2, a–d. The apex consisted of the closed rosete accompanied by two additional separated leaves (Fig. 2b). The middle segment contained the branched stem axes from the main stem up to the apex, carrying about 8–10 leaves of the main stem as shown in Fig. 2c. The lower segment consisted of the main stem, carrying 6–14 fronds, excluding the brown senescent lowest fronds (Fig. 2d). Similar segments were separated from the other species. The same plants and segments samples were first assayed for acetylene reduction and afterwards for photoacoustic signals in the photoacoustic cell.

The results obtained with segments of all three species demonstrate an unequal distribution of both photosynthesis and N_2 fixation (Fig. 3). Acetylene reduction specific activity in the lower segments was surprisingly much higher, than in the middle segments. Acetylene reduction activity was several fold higher in the lower section of both *Azolla filiculoides* and *Azolla caroliniana* and higher in the lower section of *Azolla pinnata* var. *imbricata*, compared to the middle section of these species. The number of *Anabaena* cells per unit fresh weight was counted in two species and was found to be somewhat higher in the lower segment (19,500 and 25,500 cells/mg in *A. filiculoides* and 23,500 and 28,500 cells/mg in *A. caroliniana* for middle and

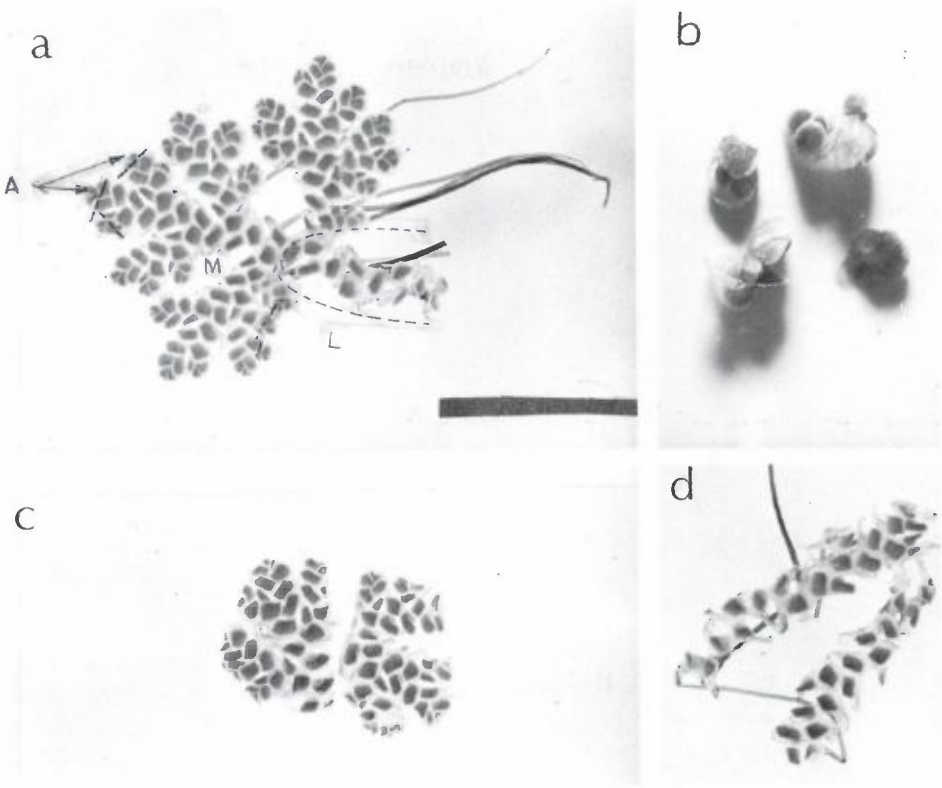

Figure 2. Whole plant of *Azolla filiculoides* and its segments. (a) Whole plant (magn. ×2.8). The segments indicated are defined by the positions against the main axis L (lower), M (middle) and A (apex). (b) Apex (magn. ×8.4) (c) Middle segment (magn. ×2.8), (d) Lower segment (magn. ×2.8). Bar = 1 cm.

lower segments respectively). Still, the increase in the heterocyst numbers per unit dry weight in the lower segment, relative to the middle, is too slight to account for the observed several fold differences of N_2 fixation activity. In contrast to the distribution of acetylene reduction activity, Fig. 3 shows that the relative O_2 evolution yield of gross photosynthesis (O/T) and the efficiency of energy storage (P.L.) are significantly higher in the middle seg-

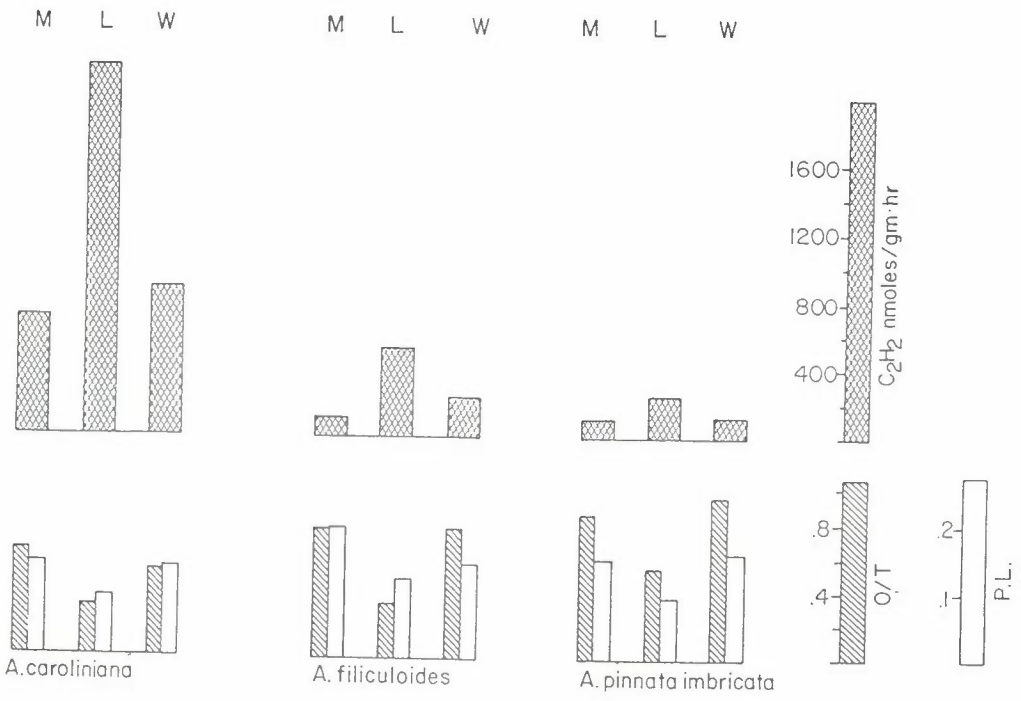

Figure 3. Histograms showing representative results of C_2H_2 reduction (top), O/T and P.L. (bottom) for the various segments of the three *Azolla* species: Middle (M), Lower (L) and also whole plants (W). Segments were prepared, and assays conducted as described in Materials and Methods. Acetylene reduction activity was expressed as nmoles C_2H_4 formed/gm fresh weight Azolla × hr.

Table 1. Distribution of activities in the different segments of *Azolla filiculoides*

Segment	No. of leaf from apex	Acetylene reduced (nmol/gm f.w hr)	O/T	PL
Upper middle	4–9	145 ± 12	1.25	0.36
Lower middle	10–14	403 ± 14	1.00	0.22
Lower part	15–24	870 ± 62	0.47	0.02

Segments were prepared, and analysis conducted as described for Fig. 3.

ments, relative to the lower segments and are usually comparable to the corresponding values for the intact *Azolla* plant.

As the lower nitrogenase activity observed in the middle segments could be due to their apical regions having young cyanobionts still undergoing differentiation, this segment was subdivided into upper and lower parts and acetylene reduction and photoacoustic measurements were conducted on them. The results (Table 1), show that nitrogenase activity increases gradually from the upper middle part to the lower middle part, confirming previous results (Hill, 1977), while photosynthesis decreases. It is clear that there is an inverse correlation between photosynthesis and N_2 fixation regarding their distribution in the plant.

The results obtained from the apices for acetylene reduction and photosynthetic activities were both very low compared to middle or lower segments, and were sometimes below detection limits. The highest values obtained from apices of *Azolla filiculoides* were 104 nmoles C_2H_4 production gr. fresh weight.$^{-1}$h^{-1} and O/T value of ca. 0.13. These segments contained only 4–5% heterocysts and the relative oxygen yield showed that photosynthetic activity in the leaves was not yet fully developed.

Accumulating considerable data in such away, it was possible to suspect that the differences in the photoacoustic results could arise from a different effect: In measuring O/T at a certain single frequency no allowance was made for the effect of damping of the modulation amplitude by the process of O_2 diffusion from the chloroplasts to the air phase (Poulet et al., 1983). Thus, the above comparison between the different segments could be obscured by possible different diffusion parameters. Hence, to ascertain the above conclusions a comprehensive study was carried out at a number of modulation frequencies. For the range of frequencies where only diffusion determines the damping dependence of O/T on the modulation frequency, the following equation can be applied (Poulet et al., 1983):

$$O/T = (O/T)_0 \exp - \left[\sqrt{\pi \ell}(1/\sqrt{D_{OX}} - 1/\sqrt{D_{th}})\sqrt{f}\right]$$

where f is the frequency of modulation, ℓ – diffusion path, (i.e. average distance from the photosynthetic membranes to the cell outskirts), D_{OX} — the oxygen diffusion coefficient and D_{th} the thermal diffusivity. Plots of ln O/T *vs.* $\sqrt{f}$ were obtained for lower and middle segments of the three *Azolla* species and were indeed linear as expected and as observed previously (Poulet et al., 1983, Canaani et al., 1984). Examples for such plots are brought in Fig. 4 for the segments of *A. filiculoides*. The slopes of these plots for the

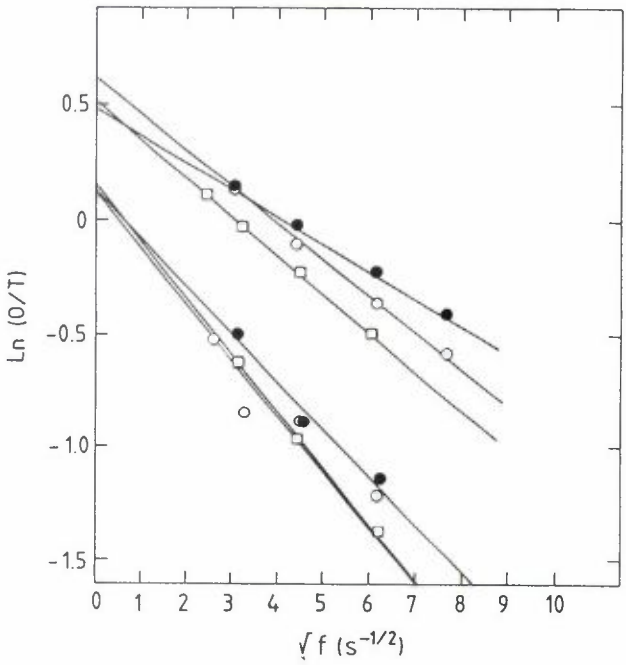

Figure 4. Dependence of Ln (O/T) on the square root modulation frequency for *Azolla filiculoides*. Top three curves — 3 different middle segment samples. Bottom three curves — 3 different lower segment samples.

lower segments are somewhat steeper than those for the middle segments, indicating some morphological differences between the two. For example,if one assumes equal diffusivities one could conclude that the O_2 diffusion path ℓ is larger in the lower segment than in the middle segment. In any case the extrapolated value of O/T for zero frequency, $(O/T)_0$, gives a measure of the true relative O_2 evolution yield, and is free of diffusion effects. Therefore, we compared $(O/T)_0$ values of middle and lower segments in the three *Azolla* species. It turns out that the extrapolated O/T is also still significantly higher in the middle segment, confirming the conclusions obtained from the measurements at a signal arbitrary frequency.

In this context it should be noted that the P.L. values are directly related to the activity of photosynthesis and confirm independently the above conclusion.

To summarize, by comparing activities of the different segments, a converse correlation was found between N_2 fixation (as measured by acetylene

reduction activity)and gross photosynthesis. Tentatively this appears to be a general trend for the different *Azolla* species, although they originate in different geographical regions and have a dissimilar morphological and anatomical structures (Lumpkin and Plucknett, 1980). The above trend was verified in a large number of repeating experiments.

Acetylene reduction activity assayed in darkness, indicated that N_2 fixation is still higher in the lower segment than the middle one (results not shown), thereby ruling out an explanation that the nitrogenase was inhibited by molecular oxygen, due to the higher photosynthetic activity in the middle segment. The disproportion between N_2 fixation and photosynthate production needs explanation, in view of the similar abundance of heterocysts. Accepting the point of view that photosynthates are directed in some proportion to serve as reductants for N_2 fixation, it seems unclear why in a specific segment where the photosynthetic activity is lower, the N_2 fixation would be higher, unless there is a mechanism which diverts a higher proportion of photosynthate to that specific segment. It is thus plausible to assume that photosynthates from the more photosynthetically active middle segment are translocated to the less photosynthetically active lower segment.

In spite of the lower specific activity of nitrogenase observed in the middle segment, it comprises the major part of the fresh weight and surface area of the frond, and therefore, the total N_2 fixation of *Azolla* occurs mostly in the middle segment. The lower segment, however, in spite of being a smaller part of the *Azolla* frond, makes also a significant contribution of 25 to 35 percent to the total N_2 fixation of the *Azolla* plant.

Our observations, conducted with a branched middle segment, *vs.* a lower segment of the main stem, were not oriented to search for the distribution of nitrogenase activity in single leaves along the main stem axes (Becking and Donze, 1981; Hill, 1975, 1977; Kaplan and Peters, 1981). The combined, experimental approach presented in this communication could be further to such analysis of single leaf activities.

Acknowledgement

This research is supported by US-Israel Cooperative Development Research Program (AID), Grant No. C5–179.

REFERENCES

Becking, J.H. and Donze, M. 1981. Pigment distribution and nitrogen fixation in *Anabaena azollae*. *Plant Soil* **61:** 203–226.

Bults, G., Horwitz, B.A., Malkin, S., and Cahen, D. 1982. Photoacoustic measurements of photosynthetic activities in whole leaves — photochemistry and gas exchange. *Biochim. Biophys. Acta* **679:** 452–465.

Buschman, C., Prehn, H., and Lichtenthaler, H. 1984. Photoacoustic spectroscopy (PAS) and its application in photosynthesis research. *Photosyn. Res.* **5:** 29–46.

Canaani, O., Ronen, R., Garty, J., Cahen, D., Malkin, S., and Galun, M. 1984. Photoacoustic study of the green alga *Trebouxia* in the lichen *Ramalina duriaei in vivo*. *Photosyn. Res.* **5:** 297–306.

Hill, D.J. 1975. The pattern of development of *Anabaena* in the *Azolla-Anabaena* symbiosis. *Planta* **122:** 179–184.

Hill, D.J. 1977. The role of *Anabaena* in the *Azolla-Anabaena* symbiosis. *New Phytol.* **78:** 611–616.

Kaplan, D. and Peters, G.A. 1981. The *Azolla-Anabaena* relationship X. $^{15}N_2$ fixation and transport in main stem axes. *New Phytol.* **89:** 337–346.

Kaplan, D., Calvert, H.E., and Peters, G.A. 1986. The *Azolla-Anabaena* relationship XII. Nitrogenase activity and phycobiliproteins of the endophyte as a function of leaf age and cell type. *Plant Physiol.* **80:** 884–890.

Lumpkin, T.a. and Plucknett, D.L. 1980. *Azolla*: botany physiology and use as green manure. *Econ. Bot.* **134:** 111–153.

Peters, G.A., Kaplan, D., Meeks, J.C., Buzby, K.M., Marsh, B.H., and Corbin, J.L. 1985. Aspects of nitrogen and carbon interchange in the *Azolla-Anabaena* symbiosis. In: *Nitrogen Fixation and CO_2 Metabolism.* P.W. Ludden and J.E. Burris, eds. Elsevier, Amsterdam, pp. 213–222.

Poulet, P., Cahen, D., and Malkin, S. 1983. Photoacoustic detection of O_2 evolution from leaves — Quantitative analysis by phase and amplitude measurements. *Biochim. Biophys. Acta* **724:** 433–446.

Watanabe, I., Espinas, C.R., Berja, N.S., and Alimagno, B.A. 1977. Utilization of the *Azolla-Anabaena* complex as a nitrogen fertilizer for rice. *IRRI Research Paper Series* **11:** 15.