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PHYSIOLOGICAL STUDIES ON N_2-FIXING AZOLLA*

G. A. Peters, O. Ito, V. V. S. Tyagi, and D. Kaplan

Charles F. Kettering Research Laboratory
150 East South College Street
Yellow Springs, OH 45387

Azolla, a genus of heterosporous aquatic ferns generally included in the *Salviniaceae*, is widely distributed in tropical and temperate fresh-water ecosystems. Members of the genus are capable of growth in environments deficient in combined nitrogen since they invariably contain an N_2-fixing cyanophyte which can provide their total N requirements. The current interest and potential of these N_2-fixing associations as an alternative N source in rice culture, as well as their long time usage for this purpose in the Far East, is well documented (Moore, 1969; Tuan and Thuyet, 1979; Liu, 1979; Singh, 1979; Rains and Talley, 1979; Talley and Rains, 1980; Watanabe et al., 1977).

The genus is generally considered to contain four new world species in the subgenus *Euazolla*, *A. caroliniana* Willd., *A. filiculoides* Lam., *A. mexicana* Presl. and *A. microphylla* Kaulfaus, and two old world species in the subgenus Rhizosperma, *A. pinnata* R.Br. and *A. nilotica* DeCaisne (Moore, 1969). The *A. nilotica* is atypical in that it is much larger than the other species (Moore, 1969; Lumpkin, pers. commun.) and the *A. pinnata* R.Br. is reported to have two morphologically distinct forms; *A. pinnata* var. *pinnata* R. Brown and *A. pinnata* var. *imbricata* (Roxb.) Bonap. (Becking, 1979). Furthermore, the *A. filiculoides* introduced to the People's Republic of China from E. Germany appears to be larger and morphologically distinct from other *A. filiculoides* (Shi et al., 1980 and Peters, unpublished observations). Based on the current interest in these associations their taxonomy would seem to merit a reassessment, and possibly a revision, using new technologies available to taxonomists.

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We have recently shown that when growth conditions are optimized, populations of A. caroliniana, A. filiculoides, A. mexicana and A. pinnata can double their biomass in two days or less and contain 5-6% N on a dry weight basis (Peters et al., 1980b). Results of comparative studies of various physiological processes in the four species are currently in preparation. These include: rates of growth, photosynthesis, light and dark respiration, N_2 fixation ($^{15}N_2$ and C_2H_2), %C, %N, etc., as a function of 12 hr, 16 hr and continuous light; the contributions of light and dark N_2 fixation; and, the effect of combined N as ammonium, nitrate and urea on physiological processes and the input from combined N and N_2 fixation. However, our most indepth studies have been, and are currently, conducted with A. caroliniana Willd. and the following account is restricted to this species.

PHOTOSYNTHESIS

The Azolla sporophyte consists of a branched floating stem bearing deeply bilobed leaves and true roots. The endophytic Anabaena occupies a specialized chamber which is formed in the aerial dorsal leaf lobes during their development (Peters, 1977; Peters et al., 1978). The Azolla contains chlorophylls a and b as well as carotenoids while the endophytic Anabaena contains chlorophyll a, phycobilins and carotenoids. The phycobilin complement is comprised of phycoerythrocyanin, phycocyanin, and allophycocyanin (Tyagi et al., 1980b). Action spectra have been obtained for photosynthesis by the association, cyanophyte-free plants and the endophyte removed from the leaf cavities (Ray et al., 1979). While the relative quantum yield is highest in the region of phycobilin absorption with the isolated endophyte, any contribution of the endophyte to the association's action spectrum is effectively masked by the preponderance of the Azolla pigments. Other studies have shown that the endophyte accounts for less than 20% of the association's chlorophyll (Peters and Mayne, 1974a; Ray et al., 1978) and the action spectra for photosynthesis by the association and endophyte-free Azolla are similar to one another and to those obtained with other green plants.

The association, fern and endophyte exhibit Calvin cycle intermediates of photosynthetic CO_2 fixation with phosphoglyceric acid being the initial product followed by hexose phosphates. Sucrose is a primary end product in the Azolla but not in the isolated endophyte (Ray et al., 1979). CO_2 fixation in the association is saturated at approximately 400 $\mu E \cdot m^2 \cdot sec$ (Ray et al., 1979) as is the growth rate (Peters et al., 1980b). The association and endophyte-free Azolla exhibit a 40% inhibition of photosynthesis (CO_2 fixation) at atmospheric O_2 as compared to 2% O_2 and an O_2 dependent CO_2 compensation point. The isolated endophyte exhibits the same, low CO_2 compensation point of about 4 ppm CO_2 at 2% and 20% O_2 and its rate of photosynthesis is also

constant at both O_2 tensions (Ray et al., 1979). Results obtained with the isolated endophyte are based on experiments in which it was isolated indiscriminately from all stages of leaf cavity formation (Peters and Mayne, 1974b; Peters et al., 1980a). As noted previously (Ray et al., 1979), since the endophyte undergoes morphological and physiological changes as a function of leaf cavity age, and since the microenvironment within the leaf cavity is still unresolved, the results obtained with the isolated endophyte demonstrate its attributes but do not necessarily extrapolate to how it functions within the Azolla leaves. The actual contributions of the individual partners to the association's total photosynthesis and photosynthesis by the association and individual partners as a function of leaf age are subjects of current investigations.

PHOTOSYNTHESIS AND N_2 FIXATION

N_2 fixation in the association and isolated endophyte has been investigated using C_2H_2 reduction, ATP-dependent H_2 production, $^{15}N_2$ fixation and their relationships (Peters and Mayne, 1974b; Peters et al., 1976, 1977). Photosynthesis is the ultimate source of all the ATP and reductant utilized in these nitrogenase-catalyzed reductions. Dark, aerobic reductions are dependent upon endogenous reserves of photosynthate, as a substrate for respiration. Rates of dark, aerobic reductions are always less than half of those obtained aerobically with light intensities saturating for N_2 fixation (Peters and Mayne, 1974b; Peters et al., 1979). This has been taken to imply that as with free-living cyanobacteria (Bottomly and Stewart, 1977; Stewart et al., 1979), respiratory driven reductions are ATP limited (Peters et al., 1980a). Dark, anaerobic reductions are negligible.

Studies in which the endogenous reserves of photosynthate have been either maintained or depleted by preincubation in the light or dark, respectively, followed by the simultaneous measurements of photosynthesis (CO_2 fixation) and N_2 fixation (C_2H_2 reduction) in the presence of DCMU (an inhibitor of Photosystem II activity) indicated the following: Photosystem II is required to provide photosynthate for reducing power but non-cyclic photophosphorylation is not a principle source of ATP for nitrogenase; CO_2 fixation can be completely inhibited by DCMU with no more than a 30% inhibition of C_2H_2 reduction if the endogenous reserves of reductant have not been depleted. Thus, the role of Photosystem II is indirect and cyclic photophosphorylation associated with Photosystem I is clearly implicated as the primary source of ATP for nitrogenase-catalyzed reductions in the light.

Action spectra of C_2H_2 reduction in the association and isolated endophyte have further demonstrated the interaction of photosynthesis with N_2 fixation (Tyagi et al., 1980b and unpublished

results). Phycobilins are generally considered to be accessory pigments for PS II, as indicated by the action spectrum for photosynthesis in the endophyte (Ray et al., 1979) and to be depleted or absent in heterocysts (Thomas, 1970; Tel-Or and Stewart, 1977; Haskelkorn, 1978). Although the above studies demonstrated that N_2 fixation is a PS I linked process not directly dependent on PS II, in the action spectra for C_2H_2 reduction in the association and the isolated endophyte, the relative rate of C_2H_2 reduction per incident quantum was essentially equal in the region of absorption by the phycobilins and that of the chlorophyll. Further, there was no appreciable affect of DCMU on the action spectrum of the endophyte and the action spectra for C_2H_2 reduction and photosynthesis differed appreciably only in that the action spectrum of photosynthesis decreased much more markedly in the region of chlorophyll absorption. At present we have no corroborating evidence for phycobilins being associated with PS I in heterocysts of the endophyte. However, there is evidence for the association of phycobilins with PS I (Wang et al., 1977) and heterocysts isolated from *Anabaena variabilis* not only contain phycobilins but the light absorbed by them (600-650 nm) is as effective in driving H_2 supported C_2H_2 reduction as the light absorbed by chlorophyll a (Peterson and Ke, 1979, and personal communication). In the case of the endophyte, attempts to isolate pure preparations of heterocysts have been frustrated by contamination with akinetes, the fact that there is a large variation in size and state of differentiation, and the simple problem of obtaining sufficient quantities of material. Current studies using fluorescence microspectroscopy of filaments of the endophyte removed from leaves of varying age have indicated that, in accord with the studies on *Anabaena variabilis* (Peterson and Ke, 1979), heterocysts of the endophyte exhibit phycobilin fluorescence (Calvert and Peters, unpublished observation).

UNIDIRECTIONAL HYDROGENASE

Previous studies (Peters et al., 1976, 1977; Peters, 1977) have shown that when the association was grown in the absence of combined nitrogen, nitrogenase-catalyzed H_2 production under Ar was generally appreciably less than the rates of C_2H_2 reduction, that H_2 production was greatest under an atmosphere of C_2H_2 and CO (see Smith et al., 1976) and that H_2 production under air was extremely low. These findings were all strongly suggestive that these associations contained a unidirectional hydrogenase as was the observation that they were efficient, in the terminology of Schubert and Evans (1976). Recent studies (Tyagi, unpublished) have shown that H_2 production is maximal under an atmosphere of 25% C_2H_2 and 0.5% CO; that preincubation under C_2H_2 for 6 hr in the light followed by assays of H_2 production and C_2H_2 reduction under the appropriate gas phase increased H_2 production up to

5-fold and C_2H_2 reduction by only 20%. The latter observation is similar to that reported for a free-living heterocystous cyanobacterium (Scherer et al., 1980) and suggestive of C_2H_2 inhibition of the unidirectional hydrogenase. H_2 production under Ar $\pm$ O_2 was also higher when photosynthesis was inhibited by DCMU, indicating that uptake may occur via the oxygen-hydrogen reaction (Peterson and Burris, 1978). Studies of the actual uptake of H_2, using 0.1% H_2 in air, have shown that after a lag period of several hours, there is appreciable H_2 uptake by the association and none in the endophyte-free Azolla and the uptake by the association is diminished by preincubation with C_2H_2. In accord with previous studies (Peters et al., 1976) there is no evidence for the occurrence of a reversible hydrogenase. These studies are continuing and details will be presented elsewhere.

N_2 FIXATION AND TRANSFER FROM THE ENDOPHYTE TO THE AZOLLA

Studies with $^{15}N_2$ have shown that the endophyte Anabaena, isolated from Azolla leaf cavities in all stages of development, releases approximately half of the N_2 it fixes into the incubation medium as ammonium with only small amounts of organic N (Peters, 1977; Peters et al., 1980a). It should be noted that more than half of the N_2 fixed may actually be released since undifferentiated filaments in these preparations may well have assimilated some of the released ammonium. Additional studies comparing the supernatants after incubations of the endophyte under $^{14}CO_2$ in Ar and in N_2 atmospheres showed ammonium under N_2 but not Ar and no difference in the ^{14}C content of the supernatants. Both contained less than 5% of the incorporated ^{14}C, further indicating that the symbiont released few or no organic N compounds such as amino acids (Ray et al., 1978; Peters et al., 1980a). In the association, incubations under $^{15}N_2$ in air followed by chase periods with air showed that there is a rapid assimilation of newly fixed N_2 into ethanol-soluble and then ethanol-insoluble fractions with a low level of free ammonia (Peters et al., 1979).

Both the fern and endophytic Anabaena were found to have the capacity to metabolize ammonium, based on the activities of glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) in each. However, about 90% of the association's GS activity and 80% of its GDH activity were attributable to the fern (Ray et al., 1978; Peters et al., 1980a). Further, since the contributions by the endophyte to the association's total activity reflected an average of its activity in all developmental stages, and a developmental gradient was known to exist (Hill, 1975, 1977; Peters et al., 1980a), it was suggested (Ray et al., 1978; Peters et al., 1979, 1980a) that activities attributed to the Anabaena might be associated with specific developmental stages, notably the undifferentiated filaments.

It has been suggested by others (Stewart, 1977; Haselkorn, 1978) that decreased GS activity in symbiotic cyanobacteria could provide an explanation for the release of ammonia with the possibility that the host modifies the endophyte's ability to assimilate ammonia by inhibiting its GS activity or synthesis. In the Azolla-Anabaena association there is evidence indicating that the endophyte's GS activity is associated with filaments in young leaf cavities and that as a function of leaf development, and increasing heterocyst frequency, the Azolla plant may actually prevent GS synthesis in the endophyte (Orr, Toan, and Haselkorn, personal communication, Third Int. N_2 Symposium, Madison, Wis.).

DEVELOPMENTAL MORPHOLOGY AND PHYSIOLOGY

General aspects of the morphology and ultrastructure of the A. caroliniana association have been presented (Duckett et al., 1975; Peters, 1977; Peters et al., 1978, 1980a). Recent studies have dealt with the developmental morphology taking advantage of the sequential leaf development. Reconstruction of serial sections of leaves of various age, as defined by their position on the stem axis (Hill, 1977), and the subsequent use of cleared and stained whole mounts, have provided new insights into the development and organization of the leaf cavity (Calvert and Peters, 1979). Of particular interest is the finding that the epidermal hairs which line the leaf cavity are of two types, simple and branched, and that whereas the simple hairs increase in number with leaf development, the branched hairs are not only differentiated early in leaf development, but their number is restricted to two per cavity. Moreover, while the simple hairs are randomly positioned around the cavity, except for the lower quadrant, the branched hairs are always found in the same position and in close proximity to the path of the foliar trace. A detailed account of these and other aspects of leaf cavity development will be presented elsewhere (Calvert et al., in preparation).

Physiological studies have been conducted in parallel with the morphological studies. Using main stem axes containing 12 or more leaves, these studies have shown that the N content is highest in the apical region, decreasing in progressively older leaves and that the dry weight as a percent of the fresh weight exhibits a similar pattern. Moreover, the gradient in nitrogenase activity as a function of leaf age shown using C_2H_2 reduction (Hill, 1977; Peters et al., 1980a) has been confirmed using $^{15}N_2$ (Fig. 1). Employing $^{15}N_2$ followed by varying chase periods with air, and a rather involved experimental protocol which will be presented in detail elsewhere (Kaplan and Peters, in preparation), it has also been demonstrated that N_2 fixed in the older leaf cavities is transported to the apical region. Studies to determine the transported compound(s), the relationship of the hairs to the physiological processes involved, and the interaction of host-

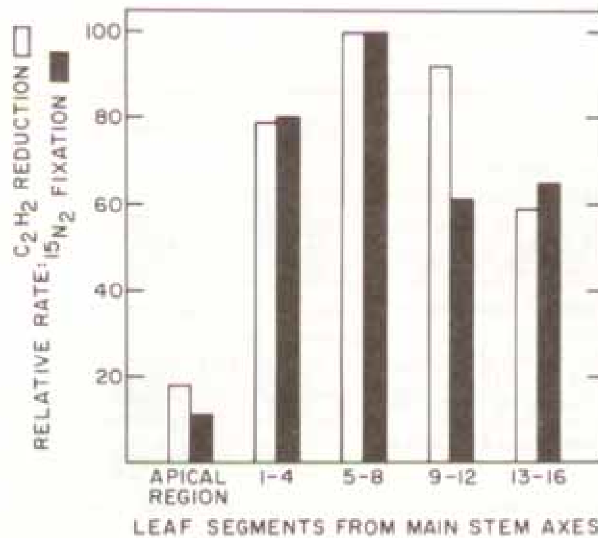

Fig. 1. Gradient in nitrogenase activity (C_2H_2 and $^{15}N_2$ reduction) as a function of leaf age in A. caroliniana.

symbiont carbon metabolism as a function of leaf age are in progress.

PHYSIOLOGICAL STUDIES USING THREE PHOTOPERIODS

Table 1 summarizes the results of studies on photosynthesis, N_2 fixation, and associated processes after two weeks growth with weekly samplings under optimized growth conditions for A. caroliniana (Peters et al., 1980b) as a function of 12 hour, 16 hour and continuous light regimes. Although the doubling times in Table 1 are slightly longer than those obtained previously (Peters et al., 1980b), they are indicative of healthy, actively growing plants.

An analysis of the data shown in Table 1 (other than that for growth and C_2H_2 reduction and H_2 production in vials), using the Duncan multiple range test at p = .05, indicated few statistically significant differences in the physiological process as a function of photoperiod. Photosynthesis in air, with and without C_2H_2, under continuous light was significantly different from the

Table 1. Photosynthesis, N_2 fixation and associated processes in *A. caroliniana* during the light interval of three photoperiods.

	Light Interval		
	12 hr	16 hr	24 hr
Doubling Time (days)	3.57± .50	2.44± .12	2.33± .12
Photosynthesis (μmoles CO_2/g fr wt·hr)			
Air	123±6	109±13	71±3
Air + 15% C_2H_2	113±5	106±14	69±5
2% O_2 - .03% CO_2 in N_2	141±4	170±21	93±10
"Photorespiration" (μmoles CO_2/g fr wt·hr)			
Photosyn. in 2% O_2 - Photosyn in air	18±8	61±27	22±11
Extrapolation to zero CO_2*	35±8	29±6	18±3
Aerobic CO_2 Compensation Point (ppm CO_2)	41±2	44±2	42±7
Dark Respiration (μmoles CO_2/g fr wt·hr)			
Air, during light period	15±3	19±6	16±1
C_2H_2 Reduction (nmoles C_2H_4/g fr wt·min)			
light, aerobic in vials	45±4	57±6	27±5
light, aerobic simultaneously with determination of photosynthesis	36±8	34±12	27±10
dark, aerobic simultaneously with respiration during light period	15±1	11±1	12±4
H_2 Production (nmoles H_2/g fr wt·min)			
light, 20% O_2 - .03% CO_2 in Ar	4.3±1.6	11.6±3.2	7±2
light, air	.2±.05	.3±.02	.0
Relative Efficiency in Light $\left(1 - \frac{H_2(O_2\text{-}CO_2\text{-}Ar)}{C_2H_4\ (C_2H_2 \text{ in air})}\right)$	.90±.01	.80±.00	.74±.00
μmoles C_2H_2 reduced/μmole CO_2 fixed	.019	.019	.023
C to N ratio	7.7±.3	-	8.8±.8

Data is the average ± S.D. of duplicate analyses in two experiments after 7 days and 14 days of growth under specified photoperiod. Growth as described in Peters et al., 1980b: IRRI medium buffered at pH 6 with 10 mM MES; constant temperature of 25°C; Light at 400 $\mu E \cdot m^2 \cdot sec$.

* Method of Decker (1957).

rates at 16 hr and 12 hr. The value obtained for "photorespiration" at the 16 hr photoperiod using the rate of photosynthesis in 2% O_2 minus that in air, was significantly different from that at 12 hr or continuous light. However, when using the method of Decker (1957) with extrapolation to zero CO_2, the value obtained for the plants under 16 hr of illumination was not significantly different from that obtained with either 12 hr or continuous illumination.

In general the data in Table 1 are entirely consistent with earlier comments in this manuscript in regard to photosynthesis, N_2 fixation, their interaction, and associated processes. For example, the O_2 inhibition of photosynthesis and values obtained for the aerobic CO_2 compensation point are consistent with a C3 type of photosynthesis and the contribution of photosynthetically-generated ATP is clearly indicated by comparison of the rates of C_2H_2 reduction in the light and dark simultaneously with measurements of photosynthesis and respiration. As seen in Table 1, the dark, aerobic rate of C_2H_2 reduction is only 25-40% of the rate obtained in the light. Furthermore, the data on C_2H_2 reduction versus H_2 production and the relative efficiency expressions, determined here by

$$1 - \frac{H_2(Ar\text{-}O_2\text{-}CO_2)}{C_2H_4(C_2H_2 \text{ in air})}$$

are consistent with the occurrence of a unidirectional hydrogenase. The data also show that there is no significant effect of 15% C_2H_2 on photosynthesis during the 10-15 minute assay period.

In order to assess the contributions of light and dark N_2 fixation to the plant's nitrogen budget, rates of $^{15}N_2$ fixation and C_2H_2 reduction were determined at the midpoint of the light and dark periods of the 12 hr - 12 hr and 16 hr - 8 hr light-dark photoperiods in separate experiments. As shown in Table 2, the ratio of C_2H_2 reduced to $^{15}N_2$ fixed is approximately 4 and, using the rates of $^{15}N_2$ fixation, dark, aerobic N_2 fixation is estimated to contribute 27% of the total daily N with a 12 hr dark period and 19% with an 8 hr dark period. (These studies were conducted several months after those shown in Table 1 and the rates of C_2H_2 reduction at 12 hr and 16 hr were lower and higher, respectively, than those obtained in the previous studies. Except for invoking biological variability we have no explanation for the differences. Nevertheless the data are indicative of the relative inputs during the light and dark cycles.) Although not shown, dark respiration and C_2H_2 reduction were found to remain constant throughout the 8 hr dark period following 16 hr of light but to begin to decline after about 7 hr of darkness after 12 hr of light when plants had been maintained under optimal conditions with these photoperiods for at least a week.

Table 2. Aerobic C_2H_2 reduction and $^{15}N_2$ fixation at midpoint of light and dark cycles for two photoperiods.

	12 hr - 12 hr		16 hr - 8 hr	
	light	dark	light	dark
nmoles C_2H_4/g fr wt·min	17.2±2.1	-	88.1±8.7	43.9±5.4
nmoles N_2/g fr wt·min	4.2±0.8	1.6±0.3	21.6±2.2	10.0±0.6
C_2H_2 reduced/N_2 fixed	4.07	-	4.08	4.40
% of total N fixed during 24 hr period	73	27	81	19

Growth conditions as for Table 1 except 600 $\mu E \cdot m^2 \cdot sec$. C_2H_2 was 15% in air, $^{15}N_2$ was approximately 40 atom % in air. Incubation period was 30 or 60 min under optimal growth conditions.

A. CAROLINIANA GROWN ON COMBINED N SOURCES (AMMONIUM, NITRATE, OR UREA)

Although Peters and Mayne (1974b) were unable to obtain growth of *A. caroliniana* on ammonium, this was the result of inadequate buffering capacity and pH drift of the growth medium (Ito, unpublished observation). Employing media buffered at pH 6 with 10 mM MES (Peters et al., 1980b) there is good growth of both the association and endophyte-free plants.

As shown in Table 3, growth rates on up to 2.5 mM ammonium, and in subsequent studies up to 5 mM, are comparable to those of plants grown in its absence. There is a gradual increase in chlorophyll content, free ammonia-N, soluble N and protein N and a gradual decrease in C_2H_2 reduction activity - 75% of the control at 2.5 mM - with increasing concentrations of ammonium in the growth medium. (In subsequent studies 2.5 mM NH_4^+ has resulted in up to a 50% inhibition of C_2H_2 reduction.) Table 4 shows that the ammonium absorption rate (AAR) determined with $^{15}NH_4^+$, increases modestly with increasing ammonium concentrations. While the nitrogen fixation rate (see legend to Table 4 for method of determination) decreases with increasing ammonium concentrations, there is generally a recovery of nitrogen fixation activity upon transfer to N-free medium and the ratios of C_2H_2 reduced to the estimated N_2 fixed are entirely reasonable. The ratio of AAR/(AAR + 2(NFR)), an index of the relative contribution of ammonium absorption to the total N input, indicates that

Table 3. Effect of NH_4^+ on growth, chlorophyll content, C_2H_2 reduction and the relationships of free ammonium-N, soluble N and protein N.

mM NH_4^+	Doubling Time (days)	mg Chl*	ARA†	μg NH_4-N*	μg soluble N*	μg protein N*
0	2.35±.26	.57±.02	61±13	15±9	333±103	1550±309
0.25	2.19±.22	.60±.02	57±12	16±12	334±65	1824±336
0.50	2.31±.19	.62±.03	53±11	19±10	366±71	1820±449
1.00	2.23±.29	.64±.03	48±12	24±15	385±96	1985±475
2.50	2.27±.30	.65±.04	46±13	34±20	417±104	2000±442

† nmoles C_2H_2 reduced/g fr wt·min

* per g fr wt

Protein N is Lowry protein ÷ 6.25

Growth as in Table 1 using a 16 hr light - 8 hr dark cycle. Values are average ± S.D. of duplicate or triplicate samples from 6 sampling periods during 28 days with frequent transfer to maintain ammonium concentrations at desired level. The variation between sampling periods was much greater than that within the duplicate or triplicate samples at the same sampling period.

Table 4. Ammonium absorption ($^{15}NH_4^+$) and N_2 fixation in *A. caroliniana*.

$mMNH_4^+$ for Growth and ^{15}N labelling	$^{14}NH_4^+$ in medium after $^{15}NH_4$ labelling	AAR[1]	NFR[2]	ARA[3]	ARA/NFR	AAR/AAR+2(NFR)[4]
0.25	+	6.2	21.9	82.2	3.86	.12
	-		21.3	75.0	3.52	
0.50	+	7.5	22.7	77.8	3.80	.14
	-		15.4	76.1	4.94	
1.00	+	8.7	15.8	69.5	4.96	.22
	-		20.7	66.2	3.20	
2.50	+	11.8	13.4	54.5	4.43	.30
	-		18.6	74.8	4.02	

1. Ammonium absorption rate (nmoles $^{15}NH_4^+$/g fr wt·min)
2. Nitrogen fixation rate (nmoles N_2 fixed/g fr wt·min).
3. Acetylene reduction activity (nmoles C_2H_4/g fr wt·min).
4. Index of contribution of ammonium absorption to N input. NFR is x2 since nmoles N_2 fixed = 2 nmoles NH_4^+.

After 36 days on specified NH_4^+ concentration using conditions as per Table 3, plants were transferred to medium containing the same concentration of $^{15}NH_4^+$ for two days. After removing material for ^{15}N analysis, half of the remaining material was transferred to medium with the same concentration of $^{14}NH_4$ and the other half to N free medium for two days. NFR was calculated from the dilution of ^{15}N in the plant material, correcting for ammonium absorption when ammonium was present.

at 2.5 mM ammonium, after five weeks of growth with frequent transfers, 70% of the plant's N is still derived from N_2 fixation. This is in reasonably good agreement with the finding that the average value for C_2H_2 reduction over the five week period was 75% of the control (Table 3).

The growth rate of *A. caroliniana* is comparable when grown on N_2 alone or on medium containing up to 25 mM NO_3^-. Table 5 shows that the chlorophyll content and percent dry matter remain quite constant with increasing nitrate concentration in the growth medium. While photosynthesis remains quite constant with increasing concentrations of nitrate, as do the %C and %N, there is a decrease in C_2H_2 reduction activity, especially between 10 mM and 25 mM, with the latter resulting in about a 60% decrease in C_2H_2 reduction activity relative to the control after five weeks of growth. This value is the average obtained from assays at weekly sampling periods which ranged from 45% to 65% inhibition relative to the control plants. Of note is the observation that H_2 production does not decrease in parallel with C_2H_2 reduction as a function of nitrate concentration and there is a marked decrease in the relative efficiency expression for plants grown on 25 mM nitrate. Previous studies have indicated that nitrate resulted

Table 5. Effect of NO_3^- on growth, photosynthesis, N_2 fixation and associated processes and other physiological parameters in *A. caroliniana*.

mM NO_3^-	Doubling Time (days)	mgChl / g fr wt	% Dry Matter	%C	%N	Photosyn[1]	ARA[2]	HPA[3]	RE[4]
0	2.07±.10	.45±.08	5.2±.5	43.9±.6	4.9±.6	75.9±1.1	56.6±28	10.4±6.5	.81
2.5	1.91±.13	.44±.03	5.5±.6	43.4±.6	4.7±.5	74.9±6.3	46.9±20	9.2±6.6	.80
10	2.03±.02	.46±.09	5.3±.3	43.4±.5	4.8±.3	73.8±14	42.0±16	11.7±6.4	.72
25	2.24±.02	.52±.05	5.8±.2	43.2±1.0	4.7±.2	68.0±2.4	23.0±6.5	15.8±8.3	.32

[1] Photosynthesis (μmoles CO_2 fixed/g fr wt·hr) in air.

[2] Acetylene reduction activity (nmoles C_2H_4/g fr wt·min) in air.

[3] H_2 production activity (nmoles H_2/g fr wt·min) in Ar-20% O_2 - .03% CO_2.

[4] Relative efficiency (1 - HPA/ARA).

Plants grown as in Table 1 except for 600 $\mu E \cdot m^2 \cdot sec$ light intensity, 16 hr - 8 hr photoperiod. Data is average ± S.D. of duplicate determinations at weekly sampling periods and transfers to new media over a five-week period.

in an increase in H_2 production both relative to C_2H_2 reduction and under an air atmosphere (Newton, 1976; Peters et al., 1976) and it was suggested that a product of nitrate metabolism might be altering the occurrence or activity of a unidirectional hydrogenase (Peters, 1977).

A study analogous to that shown in Table 4, using NH_4^+, is shown for nitrate in Table 6. The nitrate absorption rate, determined with $^{15}NO_3$, increases with increasing nitrate concentrations in the medium, and, as with ammonium, there is a recovery of nitrogenase activity in plants which are transferred from nitrate-containing solution to N-free medium. Although the ratio of C_2H_2 reduced to N_2 fixed in Table 6 are slightly higher than obtained with ammonium (Table 4), they are again quite reasonable. The index analogous to that employed with ammonium indicates that after five weeks of growth on 25 mM nitrate, 46% of the N input to the plants is derived from nitrate and 54% from N_2 fixation. In accord with this it was shown in Table 5 that the %N content was essentially constant at all concentrations of nitrate, including 25 mM where C_2H_2 reduction was inhibited 60%. Thus, the decrease in N_2 fixation is compensated by nitrate utilization, maintaining a constant N content in the tissue. Furthermore, the increase in H_2 production relative to C_2H_2 reduction as a function of nitrate concentration did not diminish the N content of the plants.

As with nitrate and ammonium, the growth of _A. caroliniana_ is comparable when grown on N_2 alone or on medium containing up to 12.5 mM urea (Table 7). Increasing concentrations of urea had no obvious effect on chlorophyll content or rates of photosynthetic CO_2 fixation but resulted in an increase in the percent dry matter and a decrease in C_2H_2 reduction. Relative to the average value of the control at the weekly sampling intervals over the three week study, 2.5 mM urea resulted in a 38% decrease, and 12.5 mM a 53% decrease, in C_2H_2 reduction activity. (Although not shown in Table 7, there was no significant effect of increasing urea concentrations on the %C and %N compared to the control.) There was considerable variation in the nitrogenase-catalyzed H_2 production under Ar-O_2-CO_2 between sampling periods as evidenced by the standard deviations (Table 7). However, within an individual sampling period the variability was much less. In contrast to the results obtained with nitrate, the average value for H_2 production decreased relative to C_2H_2 reduction with increasing urea resulting in a higher value for the relative efficiency. Studies are currently in progress to ascertain the consistency and significance of this observation in regard to unidirectional hydrogenase activities. It should also be noted that while the effect of NH_4^+ on H_2 production was not determined in conjunction with the studies shown in Tables 3 and 4, subsequent studies

Table 6. Nitrate absorption ($^{15}NO_3^-$) and N_2 fixation in <u>A. caroliniana</u>.

mM NO_3^- for Growth and ^{15}N labelling	$^{14}NO_3$ in medium after $^{15}NO_3$ labelling	NAR[1]	NFR[2]	ARA[3]	$\frac{ARA}{NFR}$	$\frac{NAR}{NAR + 2(NFR)}$[4]
2.5	+	3.11	9.74	42.8	4.40	0.14
	-		11.60	49.3	4.20	
10.0	+	5.77	9.43	41.0	4.37	0.23
	-		9.84	48.7	4.95	
25.0	+	10.10	5.97	25.3	4.24	0.46
	-		8.21	42.0	5.11	

1. Nitrate absorption rate (nmoles $^{15}NO_3^-$/g·fr wt·min)
2. As in Table 4.
3. As in Table 4.
4. Index of contribution of nitrate absorption to N input.

Growth conditions as for Table 5. Plants had been grown on specified NO_3^- concentration for 35 days prior to transferring to media with equivalent concentration of $^{15}NO_3$ for 4 days followed by division of material as in Table 4 for 4 more days to determine input from N_2 fixation $\pm$ NO_3 uptake.

Table 7. Effect of urea on growth, photosynthesis, N_2 fixation and associated processes and other physiological parameters.

mM Urea	Doubling Time (days)	$\frac{Mg\ Chl}{g \cdot fr\ wt}$	% Dry Matter	Photosyn[1]	ARA[2]	HPA[3]	RE[4]
0	1.96±.09	.51±.03	5.4±.4	72.1±5.0	61.6±17	13.2±4.7	.79
1.25	1.88±.08	.54±.03	5.3±.3	75.4±9.6	41.9±12	8.3±3.4	.81
2.50	1.85±.08	.49±.06	5.8±.3	70.4±9.4	38.4±8	7.2±4.3	.82
5.00	1.88±.09	.50±.06	6.5±.6	77.9±5.3	30.7±8	4.9±2.5	.85
12.50	1.89±.09	.50±.07	6.8±.7	72.3±4.7	28.8±7	3.3±5.1	.88

1. As in Table 5.
2. As in Table 5.
3. As in Table 5.
4. As in Table 5.

Growth conditions are in Table 5. Data is average ± S.D. of triplicate analyses at each of three weekly samplings.

which will be presented elsewhere have shown that the effect of ammonia is basically similar to that of nitrate.

Table 8. Urea absorption (^{15}N urea) and N_2 fixation in *A. carolininana*.

mM Urea for Growth and ^{15}N Labelling	^{14}N Urea After ^{15}N Labelling	UAR[1]	NFR[2]	ARA[3]	ARA/NFR	UAR/(UAR + 2(NFR))[4]
1.25	+	10.0	11.1	62.5	5.63	.31
	-		11.6	76.5	6.59	
2.50	+	11.3	9.9	50.5	5.13	.37
	-		10.7	68.1	6.36	
5.0	+	14.4	9.0	51.9	5.77	.44
	-		11.2	71.8	6.41	
12.5	+	18.7	7.4	42.0	5.71	.56
	-		8.7	77.2	8.91	

1. Urea absorption rate (nmoles ^{15}N-urea/g fr wt·min).
2. As in Table 4.
3. As in Table 4.
4. Index of contribution of urea absorption to N input.

Growth conditions as for Table 5. Plants had been grown on specified urea concentration for 21 days prior to transferring to medium with equivlent concentration of ^{15}N-urea for 4 days followed by division of material as in Table 4 for 4 more days to determine in part from N_2 fixation ± urea uptake.

Table 8 shows the urea absorption rate, using ^{15}N-urea, and other data analogous to that presented for ammonium (Table 4) and nitrate (Table 6). As with the other combined N sources there is a rapid recovery of nitrogenase activity upon transfer of the plant material from medium containing urea to N-free medium. The values obtained for C_2H_2 reduced to N_2 fixed, based on dilution of the ^{15}N-labeled plant material by either N_2 fixation alone or N_2 fix-

ation plus urea absorption, using the urea absorption rate determined with ^{15}N urea, are somewhat high, especially upon transfer to N-free medium. The reason for this is not known at present. The index analogous to that employed with ammonium and nitrate indicates that after 3 weeks growth on 2.5 and 12.5 mM urea, N_2 fixation provides 73% and 44%, respectively, of the plants total N input. In regard to the urea studies it should be noted that after a week's growth on medium containing a given urea concentration, up to 10% of the urea N is found in the medium as free ammonium-N. This breakdown requires the presence of Azolla but we do not as yet know whether it is associated with an extracellular urease activity, with breakdown of urea before uptake, or if the ammonium is being released from the plant after uptake of urea and the action of an intracellular urease.

CONCLUDING REMARKS

The studies of physiological processes as a function of photoperiod and the utilization of combined N sources, as ammonium, nitrate and urea, and their effect on physiological processes presented here were restricted to *A. caroliniana*. This association was employed as the test organism to work out experimental procedures for use in conjunction with comparative studies using *A. filiculoides*, *A. mexicana* and *A. pinnata* in addition to *A. caroliniana*. The results of these comparative studies are currently being prepared for publication.

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REFERENCES

Becking, J. H., 1979, Environmental requirements of Azolla for use in tropical rice production, in: "Proc. Intern. Rice Res. Inst., Nitrogen and Rice," IRRI, Los Baños, Laguna, Philippines.

Bottomley, P. J., and Stewart, W. D. P., 1977, ATP and nitrogenase activity in nitrogen fixing heterocystous blue-green algae, New Phytol., 79:625.

Calvert, H. E., and Peters, G. A., 1979, New morphological observations on the Azolla caroliniana-Anabaena symbiosis relevant to physiological interaction, Plant Physiol, S5:113.

Decker, J. P., 1957, Further evidence of increased carbon dioxide production accompanying photosynthesis, J. Solar Energy Sci. Eng., 1:30.

Duckett, J. R., Toth, R., and Soni, S. L., 1975, An ultrastructural study of the Azolla-Anabaena azollae relationship, New Phytol., 75:111.

Haselkorn, R., 1978, Heterocysts, Ann. Rev. Plant Physiol., 29:319.

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

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

Liu, C. C., 1979, Use of Azolla in rice production in China, in: "Proc. Intern. Rice Res. Inst., Nitrogen and Rice," IRRI, Los Baños, Laguna, Philippines.

Moore, A. W., 1969, Azolla: Biology and agronomic significance, Bot. Rev., 35:17.

Newton, J. W., 1976, Photoproduction of molecular hydrogen by a plant-algal symbiotic system, Science, 191:559.

Peters, G. A., 1977, The Azolla-Anabaena azollae symbiosis, in: "Genetic Engineering for Nitrogen Fixation," A. Hollaender, ed., Plenum Press, New York.

Peters, G. A., Evans, W. R., and Toia, R. E., Jr., 1976, Azolla-Anabaena azollae relationship. IV. Photosynthetically driven nitrogenase-catalyzed H_2 production, Plant Physiol., 58:119.

Peters, G. A., and Mayne, B. C., 1974a, The Azolla-Anabaena azollae relationship. I. Initial characterization of the association, Plant Physiol., 53:813.

Peters, G. A., and Mayne, B. C., 1974b, The Azolla-Anabaena azollae relationship. II. Localization of nitrogenase activity as assayed by acetylene reduction, Plant Physiol., 53:820.

Peters, G. A., Mayne, B. C., Ray, T. B., and Toia, R. E., Jr., 1979, Physiology and biochemistry of the Azolla-Anabaena symbiosis, in: "Proc. Intern. Rice Res. Inst., Nitrogen and Rice," IRRI, Los Baños, Laguna, Philippines.

Peters, G. A., Ray, T. B., Mayne, B. C., and Toia, R. E., Jr., 1980a, Azolla-Anabaena association: Morphological and physiological studies, in: "Nitrogen Fixation, Vol., II, Symbiotic Associations and Cyanobacteria," W. E. Newton and W. H. Orme-Johnson, eds., University Park Press, Baltimore.

Peters, G. A., Toia, R. E., Jr., and Lough, S. M., 1977, Azolla-Anabaena azollae relationship. V. $^{15}N_2$ fixation, acetylene reduction and H_2 production, Plant Physiol., 59:1021.

Peters, G. A., Toia, R. E., Jr., Raveed, D., and Levine, N. J., 1978, The Azolla-Anabaena azollae relationship. VI. Morphological aspects of the association, New Phytol., 80:583.

Peters, G. A., Toia, R. E., Jr., Evans, W. R., Crist, D. K., Mayne, B. C., and Poole, R. E., 1980b, Characterization and comparisons of five N_2 fixing Azolla-Anabaena associations. I. Optimization of growth conditions for biomass increase and N content in a controlled environment, Plant, Cell Environ., in press.

Peterson, R. B., and Burris, R. H., 1978, Hydrogen metabolism in isolated heterocysts of Anabaena 7120, Arch. Microbiol., 116: 125.

Peterson, R. B., and Ke, B., 1979, Presence of phycobilins in heterocysts isolated from Anabaena variabilis, Plant Physiol., S5:28.

Rains, D. W., and Talley, S. N., 1979, Use of Azolla in North America, in: "Proc. Intern. Rice Res. Inst., Nitrogen and Rice," IRRI, Los Baños, Laguna, Philippines.

Ray, T. B., Peters, G. A., Toia, R. E., Jr., and Mayne, B. C., 1978, The Azolla-Anabaena relationship. VII. Distribution of ammonia-assimilating enzymes, protein, and chlorophyll between host and symbiont, Plant Physiol., 62:463.

Ray, T. B., Mayne, B. C., Toia, R. E., Jr., and Peters, G. A., 1979, The Azolla-Anabaena relationship. VIII. Photosynthetic characterization of the association and individual partners, Plant Physiol., 64:791.

Scherer, S., Kerfin, W., and Böger, P., 1980, Regulatory effect of hydrogen on nitrogenase activity of the blue-green alga (cyanobacterium) Nostoc muscorum, J. Bacteriol., 141:1037.

Schubert, K. R., and Evans, H. J., 1976, Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts, Proc. Natl. Acad. Sci. USA, 73:1207.

Shi, T. C., Li, C. K., Wang, F. C., Chung, C. P., Chu, L. P., and Peters, G. A., 1980, Studies on nitrogen fixation and photosynthesis in Azolla imbricata (Roxb.) and Azolla filiculoides Lam., Acta Botanica Sinica, in press (in Chinese).

Singh, P. K., 1979, Use of Azolla in rice production in India, in: "Proc. Intern. Rice Res. Inst., Nitrogen and Rice," IRRI, Los Baños, Laguna, Philippines.

Smith, L. A., Hill, S., and Yates, M. G., 1976, Inhibition by acetylene of conventional hydrogenase in nitrogen-fixing bacteria, Nature, 262:209.

Stewart, W. D. P., 1977, A botanical ramble among the blue-green algae, Br. Phycol. J., 12:89.

Stewart, W. D. P., Rowell, P., Ladha, J. K., and Sampaio, M. J. A. M., 1979, Blue-green algae (cyanobacteria)-some aspects related to their role as sources of fixed nitrogen in paddy soils, in: "Proc. Intern. Rice Res. Inst., Nitrogen and Rice," IRRI, Los Baños, Laguna, Philippines.

Talley, S. N., and Rains, D. W., 1980, Azolla filiculoides Lam. as a fallow-season green manure for rice in temperate climate, Agronomy J., 72:11.

Tel-Or, E., and Stewart, W. D. P., 1977, Photosynthetic components and activities of nitrogen-fixing isolated heterocysts of Anabaena cylindrica, Proc. R. Soc. London Ser. B., 198:61.

Thomas, J., 1970, Absence of the pigments of photosystem II of photosynthesis in heterocysts of a blue-green alga, Nature, 258:715.

Tuan, D. T., and Thuyet, T. Q., 1979, Use of Azolla in rice production in Vietnam, in: "Proc. Intern. Rice Res. Inst., Nitrogen and Rice," IRRI, Los Baños, Laguna, Philippines.
Tyagi, V. V. S., Mayne, B. C., and Peters, G. A., 1980a, Action spectra of acetylene reduction in the Azolla-Anabaena association and in the isolated endophyte, Plant Physiol., S
Tyagi, V. V. S., Mayne, B. C., and Peters, G. A., 1980b, Purification and initial characterization of phycobiliproteins from the endophytic cyanobacterium of Azolla, Arch. Microbiol., in press.
Wang, T. C., Stevens, C. R. L., and Myers, J., 1977, Action spectra for photoreactions I and II of photosynthesis in the blue-green alga Anacystis nidulans, Photochem. Photobiol., 25:103.
Watanabe, I., Espinas, C. R., Berja, N. S., and Alimagno, C. B., 1977, Utilization of the Azolla-Anabaena complex as a nitrogen fertilizer for rice, International Rice Research Institute Research Paper Series, 11:1.

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