

THE AZOLLA-ANABAENA SYMBIOSIS: MORPHOLOGY,
PHYSIOLOGY AND USE^{1,2}

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ABSTRACT

Heterosporous ferns of the genus *Azolla* contain an N₂-fixing cyanobacterium, designated *Anabaena azollae*, in specialized leaf chambers. Our studies of the symbiosis *per se* have been primarily restricted to *A. caroliniana*. However, comparative studies have encompassed four of the six known *Azolla* species: *A. filiculoides*, *A. mexicana* and *A. pinnata*, in addition to *A. caroliniana*. The physiological, biochemical, morphological and ultrastructural studies which support a current hypothesis of host-symbiont interactions and structure-function relationships are described. The latter is largely based on recent studies of the ontogenetic sequence of leaf development and associated events in the symbiotic cyanobacterium. Under optimal growth conditions the four *Azolla* species double their biomass within two days and maintain an N content of 5-6% of the dry weight with N₂ as the only N source. Results of subsequent comparative studies of photosynthesis, respiration, nitrogen fixation and associated processes in *A. caroliniana* as a function of 12 h, 16 h and continuous light regimes are summarized. The daily N input from light and dark fixation is estimated for the four *Azolla* species. The effects of several combined N sources on growth and physiological processes under a 16 h-8 h light-dark regime and estimates of the N inputs from combined N sources and N₂ fixation, as determined using isotope dilution techniques, are presented. The historical and current use of this association as an alternative N source in rice culture is briefly described. Some of the more recent field studies by other workers are reviewed and the agronomic potential and limitations of *Azolla* as an alternative N source are discussed.

Azolla, a genus of heterosporous aquatic ferns generally considered to contain six extant species, is often included with *Salvinia* in the Salviniaceae (Smith, 1955; Moore, 1969). However, a number of investigators have placed it in a single genus family, the Azollaceae (cf. Moore, 1969; Lumpkin & Plucknett, 1980). The *Azolla* species almost invariably contain an N₂-fixing cyanobacterium as an endophyte. The endophyte belongs to the Nostocaceae and is generally referred to as *Anabaena azollae*

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Strasburger. It occupies specialized cavities formed in the dorsal leaf lobes of the fern and can provide the associations with their total N requirements via fixation of atmospheric N_2 .

The taxonomy of *Azolla* is based primarily upon features of the reproductive structures and the genus is divided into two sections. Subgenus *Euazolla* is characterized by three megaspore floats and contains the four new world species, *A. caroliniana* Willdenow, *A. filiculoides* Lamarck, *A. mexicana* Presl and *A. microphylla* Kaulfuss. The two old world species, *A. pinnata* R. Brown and *A. nilotica* DeCaisne, exhibit nine megaspore floats and constitute the subgenus *Rhizosperma*. *A. nilotica* is much larger than the other species and *A. pinnata* generally exhibits a triangular shape. Thus, the old world species are easily distinguished from one another and from the *Euazolla*. In contrast, the *Euazolla* species are quite similar in size and shape and collected and/or cultured material is often sterile, precluding the use of the primary taxonomic criterion for species delineation.

Azolla species are widely distributed in tropical and temperate freshwater ecosystems. Their growth is restricted to the more placid surfaces of ponds, canals, marshes and rice paddies. In such environments *Azolla* growth is not limited by the supply of combined nitrogen since this is provided by N_2 fixation. Thus, provided other nutritional factors are adequate, *Azolla* can exhibit rapid vegetative propagation in aqueous environments in which combined N levels are limiting to other aquatic organisms. In addition, the floating, flat spray growth mode of *Azolla* enables it effectively to harvest solar radiation.

The *Azolla*-*Anabaena* associations are the only plant-cyanobacteria symbioses known to have agronomic potential. While traditionally grown as a fertilizer crop for rice in Vietnam (Moore, 1969; Dao & Tran, 1979) and the People's Republic of China (Liu, 1979; Lumpkin & Plucknett, 1980), there has been a markedly increased interest in their use as an alternative N source for rice in many other countries during the past few years. This was triggered by the energy crisis of the mid-1970s. The rising cost, questionable stability and/or diminishing availability of energy supplies, the dependence of commercial fertilizer N production upon them and the already limited availability and/or affordability of commercial fertilizer N to the agricultural sector of a number of developing countries has strengthened the interest in *Azolla*. Thus, in many countries *Azolla* has undergone, or is currently undergoing, a rapid metamorphosis from water weed to crop plant.

Since the authors have had no personal experience with either *A. microphylla* or *A. nilotica*, we have restricted the information presented here to *A. caroliniana*, *A. filiculoides*, *A. mexicana* and *A. pinnata*. A generalized description of these *Azolla* sporophytes and their life cycle is followed by a detailed description of the *A. caroliniana*-*A. azollae* symbiosis, encompassing morphology, physiology, developmental and structure-function relationships and host-endophyte interactions. Next, we present the results of some comparative studies of growth and physiological processes using the four *Azolla* species. Finally, some results of other workers conducting field studies at a number of locations are briefly described to illustrate the agronomic potential, and limitations, of *Azolla* as an alternative N source.

SPOROPHYTES AND LIFE CYCLE

Sporophytes consist of prostrate, multibranched floating stems (rhizomes) covered with deeply bilobed leaves. Adventitious roots occur at branch points, extending into the aqueous environment. The pattern of branching and degree and pattern of fragmentation largely determine the shape or growth habit of the individual species under a given set of conditions. Vegetative propagation via fragmentation is facilitated by an abscission layer which occurs at the point of root and branch attachment.

The sporophyte has a dorsiventral organization. Each stem apex curves upward, away from the water surface. The apical cell is three sided with two cutting faces which produce cells in two lateral rows. Subsequent anticlinal divisions in the two rows of cells yield a transverse profile of cells arranged in quadrants of a circle. The cells in the dorsal quadrants give rise to the leaves while those of the ventral quadrants give rise to the roots and lateral branches.

The mature rhizome has the relatively simple anatomy characteristic of ferns, the vascular bundle being protostelic in organization. The adventitious roots have a sheath covering the apex and young portion; root hairs are found on the mature portion. Development of the root is extremely well ordered and has been studied in considerable detail in *A. pinnata* (Gunning et al., 1978). Leaves are borne in two lateral rows and may overlap to such an extent that they obscure the rhizome. Each leaf has two lobes. The ventral lobe is only one cell thick at its distal end, is nearly achlorophyllous, and floats on the water surface. The dorsal lobes are aerial and mature lobes have a multilayered mesophyll bordered by epidermal cells. The abaxial epidermis has many stomata and also bears single-celled papillae which appear to vary in shape and distribution with the species studied. Each mature dorsal leaf lobe has a cavity in the proximal half. The cavity is extracellular, being formed by an infolding of the adaxial epidermis. The cavity normally contains the endophytic *Anabaena* and, as described below, numerous epidermal hairs.

As noted, *Azolla* is heterosporous. Although the factors which trigger the sexual cycle, i.e. sporocarp formation, are not well resolved, sexual reproduction appears to enable *Azolla* to maintain itself under adverse conditions. Sporocarps are borne in pairs on short stalks which arise from the first ventral leaf lobe initial of a lateral branch. The two sporocarps of each pair may be megasporocarps, microsporocarps or one of each. The megasporocarps (female) are smaller than the microsporocarps (male). A mature megasporocarp contains a single megasporangium and the associated megaspore apparatus such as the floats. Mature microsporocarps contain stalked microsporangia. At maturity the sporocarps dehisce from the sporophyte. The microsporocarps fall apart, releasing massulae with glochidia. The massulae consist of a mass of microspores, from the microsporangia, embedded in a mucilaginous matrix from the sporangial wall. The glochidia are barbed bristles which become attached to the megasporocarps, anchoring the massulae to them. Under favorable conditions the megaspores in the megasporocarps germinate into female gametophytes which bear archegonia and the microspores germinate into male prothalli which produce antheridia. The latter release antherozoids which fertilize the oospores in the arche-

gonia. The embryo develops within the megasporocarp and the young sporophyte gradually pushes up through the indusium. When fertilization has occurred under water, as opposed to within moist aggregates of massulae and megasporocarps on the leeward side of ponds, the young sporophyte emerges to a point where it floats to the surface.

An amazing feature of these associations is that the symbiosis is maintained during the sexual cycle. *Anabaena* cells, presumably akinetes, are enclosed within the sporocarps during their development and, at least in the megasporocarp, retained to maturity. During development of the female gametophyte and fertilization, the *Anabaena* akinetes germinate to produce undifferentiated filaments. These filaments become associated with the apex of the developing sporophyte, thus providing continuity of the symbiosis and precluding the necessity for a free-living stage of the endophytic *Anabaena*.

MORPHOLOGY OF THE SYMBIOSIS

The synchronous development of *Anabaena* with leaf development in *Azolla* was initially reported by Hill (1977). Each upcurved apical meristem has a small colony of undifferentiated *Anabaena* filaments associated with it. The meristematic tissues and associated *Anabaena* cells are protected by an overarching of the differentiated dorsal and ventral leaf lobes and are completely isolated from the external environment. The growth rate of the apical *Anabaena* colony appears to be entirely coordinated with that of the *Azolla*.

Establishment of *Anabaena* filaments in each leaf begins in the developing leaves contiguous with the apical *Anabaena* colony. The partitioning of *Anabaena* filaments into each leaf appears to involve a specialized epidermal trichome termed the primary branched hair (PBH) (Calvert & Peters, 1981; Peters & Calvert, 1982). This hair originates from the axil of a leaf while it is still a primordium on the apical meristem. It rapidly differentiates a single stalk cell and several elongated terminal cells whose direction of growth is toward the *Anabaena* colony. Apical *Anabaena* filaments become entwined around the PBH terminal cells. As leaf development proceeds, the PBH and associated *Anabaena* filaments are next positioned at the proximal end of the depression which represents the initial stage in leaf cavity formation. Subsequently, cells around the rim of the depression become meristematic and produce epidermal cells. The epidermal cells close the cavity in a manner analogous to the closing of an iris diaphragm, engulfing the primary branched hair and *Anabaena* filaments. These events are effectively completed by the time the youngest dorsal leaf lobe is readily discernible to the naked eye (Calvert & Peters, 1981; Peters & Calvert, 1982).

Concomitant with the closing of the leaf cavity the *Anabaena* filaments begin to differentiate heterocysts, and many additional epidermal hairs begin to form on the cavity wall. One of these hairs is quite similar to the PBH except that it differentiates fewer terminal cells. It is always located on the back of the cavity depression and is termed the secondary branched hair, SBH (Calvert & Peters, 1981). All the other

hairs are morphologically distinct from the two branched hairs. They are comprised of only two elongated, generally cylindrical cells: a stalk cell and a terminal cell. These hairs constitute a population distinct from the branched hairs and are termed simple hairs (Calvert & Peters, 1981). The two branched hairs are located in similar positions in every leaf cavity, always on the path of the foliar trace, although separated from it by 2 or 3 layers of cells. In contrast, the simple hairs, which can number up to 25 in mature cavities, are randomly distributed around the cavity wall except for the lower distal quadrant.

Previous studies (Duckett et al., 1975; Peters et al., 1978), showed that epidermal hairs of the leaf cavities exhibit the ultrastructural characteristics of transfer cells (Pate & Gunning, 1972), and this has been verified for both the branched and simple hair populations (Calvert & Peters, 1981). However, recent ultrastructural studies as a function of development (Peters & Calvert, 1982 and unpubl. obs.) have demonstrated that there is a developmental aspect to the transfer cell ultrastructure. For example, transfer cell ultrastructure is present in the terminal cells and distal portion of the stalk cell of the PBH when in contact with the apical *Anabaena* colony. As leaf cavity development proceeds, two or three isodiametric cells, termed body cells, are cut off the distal end of the stalk cell, intercalary to the terminal cells. The body cells rapidly differentiate transfer cell ultrastructure while the terminal cells become vacuolated and senesce. At the stage of leaf development where the body cells exhibit transfer cell ultrastructure it is possible also to distinguish some of the cell wall elaborations associated with transfer cell ultrastructure in the two or three cell layers which separate the site of branched hair attachment on the cavity wall from the foliar trace. Furthermore, transfer cell ultrastructure is well developed in the foliar trace, especially in the xylem parenchyma, in the region of each branched hair. The simple hairs also exhibit ultrastructural changes as a function of leaf cavity development. Details of the developmental events in both hair populations are currently being prepared for publication.

As noted, Hill (1977) reported on the synchronous development of *Anabaena* with *Azolla* during leaf cavity formation. In general, our observations of the endophyte are consistent with his observations. The generative *Anabaena* filaments of the apical *Anabaena* colony are comprised entirely of vegetative cells. As leaf cavity development commences and the filaments associated with the terminal cells of the PBH become engulfed in the forming leaf cavity, heterocysts are differentiated. As the leaf matures, *Anabaena* filaments in the cavity multiply but the rate of cell division slows with increasing age. The individual *Anabaena* cells enlarge and continue to differentiate heterocysts. In a mature leaf cavity the vegetative cells of *Anabaena* are filled with photosynthetic lamellae, the heterocyst frequency is approximately 30%, and there are two branched hairs and approximately 25 simple hairs. Furthermore, whereas the *Anabaena* filaments occupy the entire lumen of the developing cavities, in the mature cavities the filaments are located around the periphery of those portions of the cavity wall bearing epidermal hairs and adjacent to photosynthetic mesophyll, i.e. all portions except the lower distal quadrant.

PHYSIOLOGICAL PROCESSES

Photosynthesis

Both the eukaryotic *Azolla* and the prokaryotic *Anabaena* are photosynthetic organisms and, in the association, their pigmentation is complementary. *Azolla* contains chlorophylls *a* and *b* as well as carotenoids, whereas *Anabaena* contains chlorophyll *a*, the phycobiliproteins phycoerythrocyanin, phycocyanin and allophycocyanin (Peters & Mayne, 1974a; Tyagi et al., 1980; Kaplan, unpubl. obs.) and carotenoids. In the association, the endophyte accounts for 10–20% of the total chlorophyll and about 16% of the total protein (Ray et al., 1978). The preponderance of *Azolla* pigments masks the absorption of *Anabaena* filaments and effectively obscures detection of any contribution from the endophyte to the action spectrum for photosynthesis. While photosynthesis by the endophytic *Anabaena* is maximal in the region of phycobiliprotein absorption, the action spectra for photosynthesis in the *Azolla*–*Anabaena* association and endophyte-free *Azolla* are virtually indistinguishable and comparable to that of other green plants (Ray et al., 1979).

Azolla and *Anabaena* both exhibit Calvin cycle intermediates of photosynthetic CO₂ fixation. Sucrose is a major fixation product in *Azolla*, but is not detectable as a labeled product in the isolated endophyte. While the association and endophyte-free *Azolla* exhibit an O₂ inhibition of photosynthesis and an O₂-dependent CO₂ compensation point, the isolated endophyte exhibits the same CO₂ compensation point at 2 and 20% O₂ and its photosynthesis is not diminished at atmospheric O₂ tensions (Ray et al., 1979).

Current studies (Kaplan et al., unpubl. data) have taken advantage of differences in temperature sensitivity, action spectra and O₂ inhibition of photosynthesis in the individual partners to estimate the individual contributions to the total photosynthetic capability of the association. Based on differences obtained with the association and endophyte-free *Azolla* under a number of experimental conditions, our initial estimate is that the endophyte contributes 6–10% of the total photosynthetic capability of the association. The contributions from the individual partners to the total photosynthetic capability as a function of the developmental profile have not been resolved.

Photosynthesis and N₂ Fixation

In the *Azolla*–*Anabaena* symbiosis there is a close relationship between photosynthesis and N₂ fixation (Peters & Mayne, 1974b; Peters, 1976; Peters et al., 1979, 1980a; Tyagi et al., 1981). Photosynthesis is necessarily the ultimate source of all the ATP and reductant required for nitrogenase activity. The endophyte is capable of dark, aerobic nitrogenase-catalyzed reduction of substrates. However, the rates obtained under these conditions are less than one-half of those obtained in the light and are dependent upon endogenous reserves of photosynthate. The rates of nitrogenase-catalyzed substrates are greater under microaerobic, light conditions than under aerobic, light conditions and the dark, anaerobic reduction of substrates is negligible.

Photosystem II activity is required to provide photosynthate for reducing power.

However, if endogenous reserves of photosynthate have been maintained by preincubation in the light, photosystem II (CO_2 fixation) can be completely inhibited by DCMU with no more than a 30% decrease in the rate of nitrogenase-catalyzed C_2H_2 reduction. In contrast, if the association is maintained in the dark to deplete reserves of photosynthate, the inhibition of PSII activity with DCMU also inhibits C_2H_2 reduction (Peters et al., 1980a). The cumulative results of a number of studies strongly indicate that reductant from endogenous reserves of photosynthate and photophosphorylation are the primary driving forces of nitrogenase activity in the light (Peters, 1975, 1976; Peters et al., 1980a). Moreover, if one assumes that the reductant is generated in the same manner in the light and dark, the diminished rates under aerobic, dark conditions versus those obtained under aerobic, light conditions in the presence of DCMU suggest that dark, respiratory-driven nitrogenase activity is ATP-limited, with the increased activities in the light attributed to ATP generated by cyclic photophosphorylation.

The strong interaction between photosynthesis and N_2 fixation has also been demonstrated by obtaining the action spectra for nitrogenase-catalyzed C_2H_2 reduction in the association and isolated endophyte (Tyagi et al., 1981). Of particular interest here are the facts that in both the association and isolated endophyte the relative rate of C_2H_2 reduction per incident quantum was as great in the region of phycobiliprotein absorption as it was in the region of chlorophyll absorption, and heterocysts of the endophyte were found to retain phycobiliprotein.

N₂ Fixation and Ammonia Assimilation

There are a number of studies in which the nitrogenase activity of *Azolla-Anabaena* has been estimated using acetylene reduction with rates expressed on the basis of fresh weight, dry weight, total N, chlorophyll or protein (Peters & Mayne, 1974b; Ashton & Walmsley, 1976; Becking 1976, 1979; Peters, 1976; Peters et al., 1976; Talley et al., 1977; Watanabe et al., 1977; Talley & Rains, 1980a & b). In general, variations in conditions of prior growth and assay methods preclude direct comparisons of data for the various species. However, Peters et al. (1980b) determined optimal laboratory culture conditions for *A. caroliniana*, *A. filiculoides*, *A. mexicana* and *A. pinnata*, and comparative physiological studies with these species have recently been published (Ito et al., 1980). At present, the nitrogenase-catalyzed reduction of acetylene, $^{15}\text{N}_2$, and protons, and their relationships, are best defined for the *A. caroliniana* association and the endophyte removed from it (Newton, 1976; Peters, 1976, 1977; Peters et al., 1976, 1977, 1979, 1980a, 1981a & b). The reader is referred to these publications for information regarding saturating substrate concentration, electron allocation, effects of unidirectional hydrogenase, and $\text{C}_2\text{H}_2/\text{N}_2$ conversion factors.

When isolated from all stages of cavity formation and incubated under $^{15}\text{N}_2$ the endophyte releases ammonium into the incubation medium (Peters, 1977; Peters et al., 1980a) and these and related studies (Ray et al., 1978; Peters et al., 1980a) indicated little or no release of any organic N compounds such as amino acids. Low but detectable levels of free ammonia were also found in the *Azolla* tissues and/or leaf cavities

(Newton & Cavins, 1976; Peters et al., 1979). Studies of ammonia-assimilating enzymes in the association and endophyte showed that while both partners had glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) activities, the host was estimated to account for at least 90% of the association's GS activities and 80% of the total GDH (Ray et al., 1978). It should be noted that while removal of epidermal hairs from a preparation of the endophyte had no effect on the GDH activities attributed to the endophyte, a contribution of the hairs to the GS activity attributed to the endophyte was not excluded. Moreover, as considered below, these values represent an average of the activities and distribution of these enzymes in all developmental stages of both partners. In accord with the preponderance of the ammonia-assimilating enzymes being associated with the *Azolla*, incubation of the association under $^{15}\text{N}_2$ -enriched air followed by chase periods with room air showed a low percentage of the total ^{15}N in the ammonia fraction with a rapid incorporation of ^{15}N into ethanol-soluble compounds (amino acids, etc.) and then ethanol-insoluble compounds (proteins, etc.) (Peters et al., 1979).

DEVELOPMENTAL PHYSIOLOGY

In the *Azolla*-*Anabaena* associations the generative *Anabaena* filaments associated with the plant apex lack heterocysts and do not exhibit nitrogenase activity. As leaf cavities are occupied by the endophyte, heterocysts are rapidly differentiated and the increase in heterocyst frequency is paralleled by an increase in nitrogenase activity. This developmental profile, originally described by Hill (1975) using *A. filiculoides*, has subsequently been confirmed in another population of *A. filiculoides* as well as *A. pinnata* (Shi et al., 1981) and studied in considerable detail in *A. caroliniana* (Peters et al., 1980a; Calvert and Peters, 1981; Kaplan & Peters, 1981). Physiological and biochemical studies of the whole association, endophyte-free plants and populations of the endophyte isolated from all stages of development necessarily reflect a composite of activities and/or processes. Studies of main stem axes, and individual leaves or segments of the axis bearing sequential groups of leaves, has provided a more refined approach to an understanding of structure-function relationships and host-symbiont interactions.

The absence of nitrogenase activity in *Anabaena* filaments associated with the plant apex requires that N_2 fixed by *Anabaena* in mature leaf cavities is transported to the apical region, meeting the nitrogen requirements of both the plant tissues and the generative *Anabaena* filaments. Interleaf interaction and the transport of fixed N has been demonstrated in main stem axes using a pulse-chase approach with $^{15}\text{N}_2$ (Kaplan & Peters, 1981). While this study clearly demonstrated that N fixed in mature leaf cavities is transported towards the apical region, the transported compound(s) have not been identified and, as yet, there is no *direct* evidence that N is transferred from the *Azolla* tissue to the generative *Anabaena* filaments. Kaplan and Peters (1981) also showed that the N content and dry matter decreased with increasing leaf age, while the C/N ratio increased.

These findings are consistent with the suggestion that filaments of the endophyte

which actively fix N_2 , specifically those in mature cavities, might have a diminished capability for metabolizing the resulting ammonia (Ray et al., 1978). Furthermore, they imply that the host exerts a control or regulation of metabolic processes in the endophyte as a function of the developmental gradient. In essence, the endophyte is caused to differentiate rapidly a disproportionate number of heterocysts and to exist in a state of metabolic idling, serving as an ammonium production facility. Although the factors responsible for diminished cell division, greatly increased heterocyst differentiation and diminished ability to assimilate the ammonia from N_2 fixation during the developmental profile in the *Azolla* endophyte are not yet resolved, there are some insights. While other plant-cyanobacterial symbioses do not exhibit a comparable developmental gradient, their endophytes do exhibit comparable heterocyst frequencies and/or the release of ammonia (Silvester, 1976; Rodgers & Stewart, 1977; Stewart, 1977). Furthermore, cyanophytes generally have low or undetectable levels of glutamine synthetase (GS) (Stewart, 1977; Haselkorn, 1978) and low GS levels have been postulated as a biochemical mechanism explaining ammonia excretion (Stewart, 1977). It has been suggested (Stewart, 1977; Haselkorn, 1978) that the host plants might produce effector substances which modify the endophyte's ammonia assimilating pathways by inhibiting its GS activity or synthesis. Ray et al. (1978) suggested that the *Azolla* endophyte's GS activity might be associated primarily with the undifferentiated filaments and Haselkorn et al. (1980), employing an antibody against the purified GS from *Anabaena* 7120, found that not only were the antigen levels of the endophytic *Anabaena* only 5–10% of those observed in a free-living isolate but that the antigen concentration was greatest in the endophyte associated with younger leaves. Thus, there is reason to suspect a gradient in the endophyte's GS, decreasing in parallel with the differentiation of heterocysts as well as epidermal hairs in the leaf cavities. This situation would enable high heterocyst frequencies, low GS, and ammonia release by the endophyte with its assimilation by the host.

While not found in appreciable quantities in free-living N_2 -fixing cyanobacteria or other symbiotic forms, appreciable levels of GDH were found in the *Azolla* endophyte, including a preparation from which the epidermal hairs were removed (Ray et al., 1978). Since GDH has an appreciably lower affinity for ammonia than does GS, Ray et al. (1978) postulated that the endophyte's GDH might be associated with those filaments occupying mature cavities and actively fixing N_2 . While ammonia released by the endophyte would normally be assimilated by *Azolla* GS, the endophyte's GDH could conceivably provide a regulatory role, enabling it effectively to reassimilate released ammonia at high intra-cavity ammonia concentrations. The amount of N_2 fixed by the endophyte which is utilized by the individual partners, and a better understanding of N metabolism in the association as a function of the developmental profile, are subjects of current research.

While the extent of interaction in fern-endophyte carbon metabolism is largely unknown, it may also vary as a function of the developmental profile. It has been suggested previously that the endophyte might exhibit photoheterotrophic (Peters & Mayne, 1974b) or mixotrophic (Peters, 1975) metabolism. Ray et al. (1979) suggested that sucrose produced by *Azolla* might serve as a reduced carbon source for the endo-

phyte in mature leaf cavities with metabolism of sucrose conceivably providing reductant for N_2 fixation. Although $^{14}CO_2$ pulse-chase and time-course studies showed that *Azolla*, but not the endophytic *Anabaena*, synthesized sucrose (Ray et al., 1979), sucrose, glucose and fructose have recently been identified as the major soluble di- and monosaccharides in the endophyte (Peters & Kaplan, 1981). This implies that the sucrose found in the endophyte is synthesized by the *Azolla*. Thus, in the *Azolla*-*Anabaena* association we would postulate a transition from photoautotrophic metabolism in generative filaments to a photoheterotrophic or mixotrophic mode of metabolism with increasing differentiation of heterocysts and an exogenous carbon source, sucrose, utilized to maintain levels of reducing power.

CURRENT HYPOTHESIS OF FUNCTIONAL ORGANIZATION

Within the association, the *Azolla* provides the endophytic *Anabaena* with a highly specialized niche. Since the endophyte is always protected by the dorsal leaf lobe of the *Azolla*, either within a defined cavity or by virtue of overarched lobes in the apical region, it is never directly exposed to the natural aquatic and atmospheric environments. Mineral nutrients and water must be taken up through the fern prior to their reaching the cyanophyte. Light energy incident on the cyanophyte has been filtered by the fern leaf tissues. While gases may freely diffuse into the leaf cavities, it is also possible that the gaseous composition of the leaf cavity is significantly different from the atmosphere. Thus, the endophytic *Anabaena* receives physical protection, required minerals and other nutrients, and adequate moisture; in general, a highly specialized environment created for it.

We limit ourselves here to our current interpretation of functional organization as it relates to interaction of N and C metabolism between the partners. It is our opinion that branched hairs may well be the principle sites of nitrogen interchange throughout the developmental sequence. The terminal cells of the primary branched hair associated with the apical *Anabaena* colony would release nitrogen to the generative filaments. The body cells, which develop and differentiate transfer cell ultrastructure as the terminal cells senesce and the endophyte differentiates heterocysts and exhibits nitrogenase activity, would function in the uptake of fixed nitrogen released by the endophyte. These body cells of the branched hair would logically contain high levels of ammonia-assimilating enzymes, specifically GS. The resulting amino acid(s) would move through the stalk cell of the branched hair and into the vascular system via the foliar trace. In our assessment the simple hairs are less likely to play any direct role in the assimilation of ammonia released by the endophyte. Rather, they may provide a conduit for the transfer of photosynthate, presumably as sucrose, from the mesophyll cells into the mature leaf cavities. The photosynthate, metabolized by the endophyte, would help to satisfy the high demand for reductant imposed by high nitrogenase activity and high heterocyst frequency.

GROWTH AND PHYSIOLOGICAL PROCESSES AS A FUNCTION OF PHOTOPERIOD,
AND INPUT FROM N₂ AND COMBINED N SOURCES

Peters et al. (1980b) determined biomass increase, C and N content, C₂H₂ reduction, percentage dry matter and chlorophyll *a/b* ratios for clones of *A. caroliniana*, *A. filiculoides*, *A. mexicana* (two separate populations) and *A. pinnata* as a function of nutrient solution, pH, temperature, photoperiod and light intensity. Controlled environment studies were supplemented by glasshouse studies. The tolerance of the individual species to elevated temperature was *A. mexicana* > *A. pinnata* > *A. caroliniana* > *A. filiculoides*. Using the optimum growth temperature, a 16 h light period and a photon flux density of at least 400 $\mu\text{E}/\text{m}^2 \cdot \text{sec}$, the *Azolla* species were shown to double their biomass in 2 days or less and contain 5–6% N on a dry weight basis with N₂ fixed by the endophyte as the only N source.

Table I summarizes the results from subsequent studies of photosynthesis, N₂ fixation and associated processes after two weeks of growth with weekly samplings under the optimal growth conditions for *A. caroliniana* as a function of 12 h, 16 h and continuous light regimes. The doubling times are slightly longer than those obtained previously (Peters et al., 1980b), but are indicative of actively growing plants. In general, the results obtained with *A. filiculoides*, *A. mexicana* and *A. pinnata* were similar to those obtained with *A. caroliniana*.

The data in Table I are consistent with earlier comments in this manuscript in regard to photosynthesis, N₂ fixation, their interaction, and associated processes. For example, the O₂ inhibition of photosynthesis and values obtained for the aerobic CO₂ compensation point are consistent with a C₃-type of photosynthesis and the contribution of photosynthetically-generated ATP is indicated by comparison of the rates of C₂H₂ reduction in the light and dark simultaneously with measurements of photosynthesis and respiration. As shown, the dark, aerobic rate of C₂H₂ reduction is only 25–40% of the rate obtained in the light. Furthermore, the data on C₂H₂ reduction versus H₂ production are consistent with the occurrence of a unidirectional hydrogenase. The data in Table I also show that there is no significant effect of 15% C₂H₂ on photosynthesis during the 10–15 min assay period.

In conjunction with the type of studies shown in Table I, photosynthesis and dark respiration have been monitored simultaneously with C₂H₂ reduction throughout the light and dark portion of the 12 h–12 h and 16 h–8 h light–dark photoperiods and ¹⁵N₂ reduction determined at the midpoint of the light and dark period for the four *Azolla* species (Ito et al., 1980). While the changes in rates of photosynthesis, respiration, and C₂H₂ reduction as a function of the time in light or dark are not shown here, Tables II and III show the C₂H₂/N₂ conversion factors and our estimates of the daily N input during the light and dark for the four *Azolla* species, respectively (Ito et al., 1980).

The effect of several concentrations of ammonia, nitrate and urea on growth rates, dry matter content, %C, %N and the estimated percentage of the daily N inputs from the combined N source as well as the relative rates of C₂H₂ reduction for the four *Azolla* species using a 16 h–8 h light-dark regime are shown in Table IVa–c. The

TABLE I
Growth, photosynthesis, nitrogenase activity and associated processes for
A. caroliniana grown under three light regimes¹

	Light interval (h)		
	12	16	24
Doubling time (days)	3.64 ± 0.56	2.43 ± 0.09	2.36 ± 0.09
Photosynthesis (PS) (μmoles CO ₂ /mg chl·h)			
Air	217 ± 16	195 ± 30	142 ± 10
Air + 15% C ₂ H ₂	197 ± 16	190 ± 28	138 ± 13
2% O ₂ - 0.03% CO ₂ - Balance N ₂	236 ± 34	303 ± 42	186 ± 29
"Photorespiration" (μmoles CO ₂ /mg chl·h)			
PS in 2% O ₂ - PS in Air	33 ± 14	108 ± 46	45 ± 25
Extrapolation to zero CO ₂	60 ± 14	52 ± 9	37 ± 9
Aerobic CO ₂ compensation point (ppm CO ₂)	41 ± 2	44 ± 2	42 ± 7
Dark respiration (R) (μmoles CO ₂ /mg chl·h)			
Air, during light period	26 ± 5	34 ± 9	32 ± 4
C ₂ H ₂ reduction (nmoles C ₂ H ₄ /mg chl·min)			
Light, aerobic in vials	72 ± 12	100 ± 13	51 ± 5
Light, aerobic, simultaneous with PS	63 ± 17	61 ± 23	57 ± 19
Dark, aerobic, simultaneous with R	26 ± 4	20 ± 1	24 ± 8
H ₂ production (nmoles H ₂ /mg chl·min)			
Light, 20% O ₂ - 0.03% CO ₂ - Balance Air in vials	8 ± 3	20 ± 6	14 ± 4
Light, aerobic in vials	0.27 ± 0.09	0.57 ± 0.13	0 ± 0
Chl content (mg/g fr wt)	0.58 ± 0.03	0.56 ± 0.02	0.50 ± 0.05
Chl a/b	3.86 ± 0.18	4.33 ± 0.09	3.54 ± 0.10
% Dry matter	5.4 ± 0.3	-	6.4 ± 0.1
% C	42.9 ± 1.0	-	42.1 ± 0.8
% N	5.5 ± 0.3	-	4.8 ± 0.5

¹ Values shown are the average of 4-12 determinations.

results of preliminary studies conducted with *A. caroliniana* have already been presented (Peters et al., 1981a). Detailed results of these studies showing effects of the combined N sources on CO₂ exchange rates in the light and dark, actual rates of C₂H₂ reduction and H₂ production, absorption rates of the combined N sources and estimated N₂ fixation rate using isotope dilution techniques along with the type of information presented in Table IV will be presented elsewhere (Ito & Peters, in prep.).

Azolla as an Alternative N Source for Rice

Azolla has a long history of use as a fodder and/or green manure crop for rice culture in Vietnam and the People's Republic of China (Moore, 1969; Dao & Tran,

TABLE II
Ratio of C_2H_2 reduced to ^{15}N fixed during the day (D) and night (N) of two photoperiods¹

Photoperiod		<i>A. caroliniana</i>	<i>A. filiculoides</i>	<i>A. mexicana</i>	<i>A. pinnata</i>
12 h-12 h	D	2.15	2.48	4.14	3.79
	N	3.28	2.57	5.04	5.26
16 h-8 h	D	4.09	4.00	4.61	3.69
	N	4.40	3.58	4.67	3.73

¹ C_2H_2 reduction determined under 15% C_2H_2 in air, and $^{15}N_2$ reduction determined under 80% N_2 , 20% O_2 , 0.03% CO_2 .

TABLE III
Contribution of N_2 fixation during the light period and during the dark period to the total daily N_2 fixation¹

Species	Photoperiod			
	12 h-12 h		16 h-8 h	
	Light	Dark ²	Light	Dark
<i>A. caroliniana</i>	72	28	81	19
<i>A. filiculoides</i>	85	15	81	19
<i>A. mexicana</i>	82	18	85	15
<i>A. pinnata</i>	85	15	82	18

¹ Based on $^{15}N_2$ fixation rate determined at midpoint of light and dark cycles of the two photoperiods.

² The input during the dark period of the 12 h-12 h photoperiod may be slightly overestimated since C_2H_2 reduction declines after 7 h.

1979; Liu, 1979; Lumpkin & Plucknett, 1980). Field studies initiated five to six years ago at the International Rice Research Institute (IRRI) in the Philippines (Watanabe et al., 1977), the Central Rice Research Institute (CRRI), Cuttack, India (Singh, 1977), the University of California, Davis (Talley et al., 1977), and Sri Lanka (Kulasooriya & de Silva, 1977) to determine the applicability of *Azolla* in rice production have confirmed its potential as an alternative or supplemental nitrogen source for rice in tropical as well as temperate regions. *Azolla* is most effective when used as a green manure, but nitrogen is also provided when it is grown as a cover crop with rice (Talley et al., 1977; Singh, 1979a & b). In California, *A. mexicana* was more effective in increasing rice yields when grown in dual culture than *A. filiculoides* (Talley et al., 1977; Talley & Rains, 1980a), and a combination of *Azolla* grown in dual culture with rice following incorporation of *Azolla* grown during the fallow season increased yields relative to those obtained with the incorporated *Azolla* alone. In addition, *Azolla* used in dual culture had the added benefit of suppressing weed growth. At IRRI, the total N requirements of a rice crop can be provided using the

TABLE IVa-c
Effect of Ammonium (a), nitrate (b) and urea (c) on growth, % dry matter, %C, %N, the estimated percentage of daily N input from the combined N source, and the relative rate of C_2H_4 reduction^a

	<i>A. caroliniana</i>			<i>A. filiformis</i>			<i>A. mexicana</i>			<i>A. phoenicea</i>		
	0	2.5	5.0	0	2.5	5.0	0	2.5	5.0	0	2.5	5.0
(a)												
mM NH ₄ in growth medium	1.85 ± 0.03	1.78 ± 0.05	1.84 ± 0.07	1.75 ± 0.04	1.84 ± 0.06	1.75 ± 0.04	1.97 ± 0.32	1.86 ± 0.12	1.91 ± 0.13	1.97 ± 0.13	1.84 ± 0.13	1.85 ± 0.20
Doubling time (days)	5.8 ± 0.4	5.5 ± 0.2	5.7 ± 0.3	5.4 ± 0.2	5.7 ± 0.2	5.7 ± 0.2	5.4 ± 0.8	5.6 ± 0.8	5.9 ± 1.0	5.9 ± 0.6	6.0 ± 0.3	5.8 ± 0.1
% Dry matter	43.2 ± 0.1	43.2 ± 0.2	43.5 ± 0.5	43.1 ± 0.1	43.3 ± 0.8	43.6 ± 0.4	42.3 ± 0.8	43.8 ± 0.9	44.6 ± 0.6	42.3 ± 0.5	44.1 ± 0.6	44.2 ± 0.1
%C	4.6 ± 0.3	5.5 ± 0.1	5.6 ± 0.3	5.6 ± 0.4	6.4 ± 0.1	6.3 ± 0.2	6.1 ± 0.2	6.7 ± 0.4	6.8 ± 0.4	4.6 ± 0.2	5.4 ± 0.4	6.0 ± 0.5
% of daily N input from NH ₄	0	35	52	0	61	63	0	37	45	0	41	47
Relative C_2H_4 reduction	100	61	57	100	58	49	100	67	62	100	54	49
(b)												
mM NO ₃ in growth medium	0	10	25	0	10	25	0	10	25	0	10	25
Doubling time (days)	2.16 ± 0.14	2.08 ± 0.17	2.18 ± 0.12	1.97 ± 0.31	2.01 ± 0.06	2.00 ± 0.05	1.93 ± 0.08	1.93 ± 0.08	2.07 ± 0.07	1.91 ± 0.04	1.98 ± 0.08	2.28 ± 0.15
% Dry matter	5.6 ± 0.7	5.7 ± 0.8	6.3 ± 0.8	4.8 ± 0.4	5.3 ± 0.6	5.7 ± 1.0	4.8 ± 0.4	5.1 ± 0.5	5.2 ± 0.4	4.9 ± 0.8	5.2 ± 0.6	5.2 ± 0.7
%C	42.8 ± 0.1	42.7 ± 0.1	42.3 ± 0.1	42.0 ± 0.4	42.0 ± 0.3	41.3 ± 0.4	43.0 ± 0.9	42.3 ± 0.7	40.9 ± 1.0	43.4 ± 0.2	43.3 ± 0.6	43.0 ± 0.7
%N	5.1 ± 0.7	5.3 ± 0.5	4.7 ± 0.7	6.0 ± 0.8	6.1 ± 0.7	6.0 ± 0.6	6.6 ± 0.5	6.8 ± 0.4	6.5 ± 0.5	5.6 ± 0.9	5.7 ± 0.5	6.1 ± 0.4
% of daily N input from NO ₃	0	25	63	0	21	31	0	9	17	0	16	24
Relative C_2H_4 reduction	100	66	38	100	61	41	100	100	80	100	77	58
(c)												
mM urea in growth medium	0	1.25	12.5	0	1.25	12.5	0	1.25	12.5	0	1.25	12.5
Doubling time (days)	2.04 ± 0.12	1.92 ± 0.46	1.89 ± 0.08	1.92 ± 0.07	1.93 ± 0.09	1.83 ± 0.03	1.81 ± 0.06	1.82 ± 0.12	1.88 ± 0.08	1.87 ± 0.03	1.81 ± 0.04	1.84 ± 0.06
% Dry matter	5.3 ± 0.5	5.6 ± 0.8	6.1 ± 1.1	4.9 ± 0.7	4.9 ± 0.2	5.4 ± 0.2	4.6 ± 0.4	4.7 ± 0.6	5.1 ± 0.1	4.8 ± 0.3	5.2 ± 0.7	5.4 ± 0.7
%C	41.3 ± 1.1	42.8 ± 0.9	43.4 ± 0.8	41.2 ± 0.5	42.6 ± 0.5	42.6 ± 0.2	42.7 ± 1.5	42.6 ± 1.3	43.2 ± 0.9	42.5 ± 1.2	42.8 ± 1.3	43.2 ± 1.1
%N	5.5 ± 0.2	5.7 ± 0.6	6.2 ± 0.7	5.8 ± 0.6	6.6 ± 0.3	7.0 ± 0.4	7.0 ± 0.2	7.3 ± 0.4	8.1 ± 0.6	5.7 ± 0.4	6.0 ± 0.5	7.0 ± 0.2
% of daily N input from urea	0	20	41	0	22	51	0	23	50	0	25	49
Relative C_2H_4 reduction	100	94	52	100	67	43	100	80	42	100	61	50

^a The plants were grown under optimal conditions with the indicated concentration of the combined N source for 3 weeks with weekly transfers. Except for the estimate of the daily N input, the plants were sampled weekly, in duplicate, and the data reflects the average value of all determinations. Estimates of the daily N input are based on duplicate determinations, using isotope dilution techniques at the end of the 3-wk period. The relative C_2H_4 reduction is expressed as a percentage of the control grown in the absence of combined N, simply using the mean values from the total study period.

Chinese dual row method and a more labor intensive approach where *Azolla* is incorporated into the paddy soil at intervals throughout the growing season. Other studies at IRRI have shown an accumulation of 330 kgN/ha over 220 days (Watanabe et al., 1977) and 460 kgN/ha from 22 consecutive crops of *A. pinnata* over 330 days with an average daily accumulation of 1.4 kgN/ha (Watanabe, 1978). In fallow paddies in California inoculation with *A. filiculoides* equivalent to 1.2 kgN/ha has given yields between 33 and 93 kgN/ha (Talley & Rains, 1980b). In a demonstration of the relationship between biomass and nitrogen content in *A. filiculoides* growing in a fallow rice field in California, Talley and Rains (1980a) showed that an inoculum of 50 gm fresh wt/m², giving about 10% surface cover, attained full cover in 15 days. Twenty days later the mature cover had a biomass of 1700 kg dry wt/ha and a nitrogen content of 52 kgN/ha. *Azolla* N and commercial ammonium sulfate in the range of 30–60 kgN/ha give equivalent rice yields and the effect of the N sources are additive (Singh, 1977; Talley et al., 1977; Talley & Rains, 1980b). At higher N levels the *Azolla* N appears to be less effective, possibly due to slower mineralization or loss through denitrification. A diminished availability of *Azolla* N at high N levels has also been found using pot studies (Watanabe et al., 1977), and ammonia release was more rapid from fresh *Azolla* than dried *Azolla*. Mineralization was relatively slow, with 6–8 wk required for 75% of the *Azolla* N to become available. Singh (1979b) also found that *Azolla* nitrogen was released rather slowly. He reported that under flooded conditions at 24 ± 2°C 34%, 56% and 82% of the *Azolla* N was released after 1, 3 and 6 wk, respectively, while at room temperature 80% of the *Azolla* N was released after 3 wk. In India, the average annual production of *Azolla* over three years at CRRRI, was about 347 tons fresh wt/ha containing 868 kgN (Singh, 1979a, b), obtained from 12–15 crops harvested at 20–30 day intervals. A single crop of *A. pinnata* incorporated into rice fields provided the equivalent of at least 20–30 kgN/ha of chemical fertilizer N. When several *Azolla* crops or dual cropping were used, the equivalent of 50 kgN/ha was added and rice yields increased up to 40%, 0.5–2 tons/ha, versus unfertilized controls (Singh, 1979a & b; Singh, 1980 and pers. commun.). Moreover, as shown above, *Azolla* retains nitrogenase activity in the presence of combined N fertilizer, its N contribution is additive to the chemical fertilizer N, and it adds large quantities of organic matter to the soil, thus both enriching it and improving its texture.

In the last few years the IRRI has provided impetus to *Azolla* field research. IRRI currently conducts training programs on *Azolla* and its utilization with rice and sponsors an international collaborative field trial on the effect of phosphorus on growth, N, and P content of selected *Azolla* isolates in areas including Thailand, Nepal, Bangladesh and India. *Azolla* species, including the isolates from widely separated geographical areas, are being characterized in regard to temperature tolerances, pest resistance, and N input, selecting those best suited for a specific environment.

It is obvious that *Azolla* has potential as an alternative N source for rice in many areas. Nevertheless, much remains to be determined and *Azolla* is by no means a panacea. Although considerable attention has been focused on the phosphate requirement of *Azolla* no attempt has been made here to review the literature on this topic.

Rather, the authors simply note the following: (1) It is important to realize that the phosphate requirement may well vary with local, overall management practices and other factors; (2) One of the problems with adding phosphate to *Azolla*, and a reason for employing split applications, is that the phosphate rapidly becomes unavailable to the floating *Azolla*. Actually, the phosphate requirement of *Azolla* might best be met by the advent of a slow release source which either floats or has a specific attraction to the *Azolla* sporophyte; (3) There is reason to believe that *Azolla* strains which have even lower phosphate requirements may be found through screening procedures. For example, an *A. pinnata* isolate from Vietnam reportedly grows well in paddies at CRRRI without application of phosphorus (Singh, 1980); (4) While added phosphorus becomes tied up in the paddy soil, this phosphate is available to the rice plants as is the phosphate taken up by *Azolla* (cf. Talley & Rains, 1980b).

Azolla is also subject to attack by pests, especially larvae of various lepidopterous and dipterous insects which can do serious damage in a very short time. These pests can be controlled by pesticides such as carbofuran, and here again it may be possible to select *Azolla* strains which are more resistant than others to insect predation. Although *Azolla* species/strains are not known to carry any rice diseases or insect pests, knowledge in this area is still limited. Until this has been absolutely established, caution is warranted. At the very least, when *Azolla* stocks are transferred from one geographical area to another the stocks should, in our opinion, be fumigated and/or surface sterilized before conducting field studies to guard against the inadvertent introduction of a new pest or rice disease.

The water requirement of *Azolla* necessarily limits its potential use as a green manure grown during the fallow season in some areas since the water is simply not available. Moreover, there is no definitive information on the extent to which *Azolla* may compete with rice for water in a dual culture situation and this clearly needs to be ascertained.

In a recent study of the effect of three insecticides and three herbicides on growth and physiological processes in four *Azolla* species (Toia et al., 1981), the *Azolla* was found to be quite sensitive to Butachlor (Machete EC) and Propanil (STAM F-34). Thus, undesirable *Azolla* growth can probably be readily controlled in localized situations where it might pose a problem. Herbicides may also have an application in conjunction with *Azolla* in dual culture with rice. For example, rice is relatively tolerant to Propanil while *Azolla* is killed by a solution of 1 ppm. Thus, depending upon local management practices, it may be possible to use a herbicide to kill off the *Azolla* selectively at the tillering stage of rice, timing the N input from the total *Azolla* biomass and decreasing the amount of labor required to get the *Azolla* N to the rice plants.

At present the use of *Azolla* is indisputably labor intensive. Maintenance ponds are required for multiplication of the inoculum for the field and the weight of the fresh material and its fragile nature necessarily preclude any long distance transport. In the future it may be possible to employ *Azolla* spores for an inoculum in much the same manner as a seed. This will be largely dependent upon our increased understanding of those factors which trigger sporulation, such that it can be controlled — i.e. caused

or prevented. This information is, in our opinion, quite central to the future of *Azolla*. If sporulation can be induced, a method developed for harvesting the spores, and the spores stored for a prolonged period, a number of obstacles will be overcome and new vistas opened.

In conclusion, the consideration of *Azolla* as an agronomic crop is taking hold. While there are problems associated with its widespread use these may be at least partially alleviated by future research. As with the improvement of other plant species this will necessitate laboratory study, field testing, further refinement of management practices and possibly some mechanization through the design of mechanical equipment specifically adapted for use with *Azolla* in rice.

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