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The *Azolla-Anabaena* Association: Historical Perspective, Symbiosis and Energy Metabolism

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I. Abstract

The heterosporous water-fern genus *Azolla* is one of the few symbioses with a cyanobacterium in the genus *Anabaena.* The *Azolla-Anabaena* association includes six extant species of *Azolla,* which are widely distributed in relatively placid tropical and/or temperate freshwater environments.

The earliest mention of the plant seems to be in an ancient Chinese dictionary that appeared about 2000 years ago. *Azolla* was used in about the 11th century in Vietnam. By 1980 renewed interest in this symbiotic association was shown by the demand for a less fossil energy-dependent agricultural technology. The importation of a variety of *A. filiculoides* may have been a most significant breakthrough for the improvement of *Azolla* cultivation in China. The history of research may be divided into three periods and a new biotechnological stage of *Azolla* research has recently begun.

Each mature dorsal leaf lobe has an ellipsoid cavity which contains *Anabaena azollae* throughout its development. Heterocystous *A. azollae* from six *Azolla* species share identical and highly specific antigens. *Azolla* and its endophyte exhibit a coordinated pattern of differentiation and development. Epidermal hair cells of the host are probably interactive with the symbiont. The interior surface of a mature leaf cavity is lined with an envelope and covered by a mucilaginous layer. *A. azollae* shares the cavity with small populations of the bacteria *Pseudomonas* and *Azotobacter.* Endophyte-free *Azolla* may rarely occur in nature and can be generated by aseptic techniques. *Anabaena azollae* can be isolated from *Azolla* fronds by gentle pressure and by enzymatic digestion. The free-living cultures derived from the *Anabaena* so obtained differ in some respects, however, from the freshly extracted symbiont, and might better be called the presumptive isolate.

Both *Azolla* and *Anabaena* contain specific photosynthetic pigments. The optimum conditions for photosynthesis have been measured. *Azolla* is a C_3 plant and has high net photosynthesis. PSII activity in the symbiont is low. Nitrogenase is localized in the heterocysts of the symbiont and has some advantages compared with free-living cyanobacteria. Symbiotic *A. azollae* has a high frequency of heterocysts. Unidirectional hydrogenase occurs in the symbiont and recycles electrons and ATP. Simultaneous measurements of N_2 fixation and photosynthesis show the dependence of nitrogenase on photosynthetically captured radiation for energy by an indirect dependence on CO_2 fixation. The host contains most of the total GS and GDH activities, and the symbiont excretes a substantial portion of its newly fixed nitrogen as ammonium. The two partners in the association exhibit a comparable developmental gradient and a mechanism

of cooperative integration for their energy metabolism, thus improving the efficiency of solar energy conversion and presenting a unique model for biotechnology.

Sumario

El género acuático *Azolla* es una de las pocas plantas simbióticas cianobacterianas, dentro del género *Anabaena.* El género *Azolla-Anabaena* incluye seis especies bastante bien conocidas, ampliamente distribuidas en zonas tropicales apacibles y en ambientes de aguas templadas.

El data mas antiguo que se tiene de ésta planta parece orginarse de un diccionario chino hace unos 2000 años. *Azolla* ya se utilizaba en Vietnam por el siglo XI. Para el año 1980 esta asociación simbiótica habia despertado un gran interés, estimulado por la necesidad de reducir la dependencia energética del petróleo en el sector agrario.

La introducción de una variedad de *A. filiculoides* parece haber sido uno de los pasos mas decisivos en el mejoramiento del cultivo de ésta planta en China. Historicamente el estudio científico de la *Azolla* puede dividirse en tres fases principales, junto con una reciente etapa de investigación biotecnológica.

La parte interior de la hoja madura tiene una cavidad elíptica que contiene *Anabaena azollae* a lo largo de su desarrollo. *A. azollae* orginaria de seis especies diferentes de *Azolla,* comparten antigenos idénicos y altamente especializados. La actividad endófica de la *Azolla* exibe un cierto patron de coordinación y desarrollo. Los pelillos epidérmicos celulares del tronquillo son probablemente interactivos con la simbioses. La parte interior ellíptica de la hoja madura está cubierta de una tela mucosa. *A. azollae* comparte esta cabidad con una población pequeña de bacterias *Pseudomonas* y *Azotobacter.* "Endophyte-free" *Azolla* raramente se da en la naturaleza, pudiendo ser generada por medio de técnicas asépticas. La separación de *A. azollae* puede otenerse presionando suavemente éstas o por medio de digestión enzimática, y se ha demostrado por medios inmunológicos y patrones de hibridización que estos no están estrechamente relacionados a la simbiosis de la planta.

Tanto *Azolla* como *Anabaena* contienen ciertos pigmentos fotosintéticos específicos. Las condiciones óptimas fotosintéticas de la *Azolla* han sido ya calculadas. *Azolla* es una planta C_3 con alta capacidad fotosintética. La actividad de PSII en la simbiosis es baja. El nitrógeno se localiza en areas heterocistas simbióticas, ofreciendo ciertas ventajas comparadas con cianobacteria libre. La simbiosis de *A. azollae* posee una alta frecuencia heterocista. La hidrogenización occurre en la simbiosis, reciclando electrones y ATP. Trabajos realizados simultaneamente para medir la fijación de nitrógeno y de fotosíntesis, muestran la dependencia del nitrógeno en

la radiación capturada fotosinteticamente por la energia y una dependencia indirecta en cuanto a la fijación de CO_2. La parte troncular contiene la mayor parte de las actividades activas de GS y GDH, mientras que la simbiosis excreta una parte substancial de su nitrógeno nuevamente fijado como amonio. Este acoplamiento exibe un gradiente de desarrollo y un mecanismo de integración conjunto y de cooperación substancial en su metabolismo energético, a fin de mejorar su eficacia en la conversión de energía solar, por lo cual presenta un modelo único para la biotecnología.

II. Introduction

Azolla is a genus of small aquatic ferns of demonstrated importance to the agriculture of developing countries (Lumpkin & Plucknett, 1982; Moore, 1969; Shi, 1981; Watanabe, 1982). Recently, the interest in this fern-cyanobacterium association has been renewed by the demand for a less fossil energy-dependent agriculture technology.

A. PLANT-CYANOBACTERIA SYMBIOSES

Cyanobacteria are almost exclusively free-living forms, but a few species form specific associations with various plant groups. There are some 500 genera and 17,000–18,000 species of lichens, and 8% of them contain a cyanobacterium as a phycobiont (Fogg et al., 1973; Millbank, 1974; Stewart et al., 1980, 1983). The cyanobionts associate with six species of one pteridophyte genus (*Azolla*), some species of two diatom genera (*Rhizosolenia* and *Rhophalodia*), about five liverwort genera (e.g., *Anthoceros, Blasia,* and *Cavicularia*), 90 species of nine genera in one group of gymnosperms (the cycads) and about 40 species of one angiosperm genus (*Gunnera* in the Halagoraceae) (Peters et al., 1986; Sprent & Raven, 1985; Stewart et al., 1977, 1980, 1983). Such associations occur with representatives from a broad segment of the plant kingdom; however, the cyanobacterium in the symbiosis is always a member of the Nostocaceae, a family characterized by the ability of its members to differentiate heterocysts and fix atmospheric N_2.

The applied significance of the known plant-cyanobacteria symbioses has so far rested on only the *Azolla-Anabaena azollae* symbiosis (Stewart, 1982).

B. LIFE CYCLE

A generalized life cycle for *Azolla* is shown in Figure 1 (Lucas & Duckett, 1980; Lumpkin & Plucknett, 1980; Peters & Calvert, 1983).

Azolla spp. are heterosporous ferns. Sporocarps are borne in pairs on short stalks that arise from the first ventral leaf lobe initial of a lateral

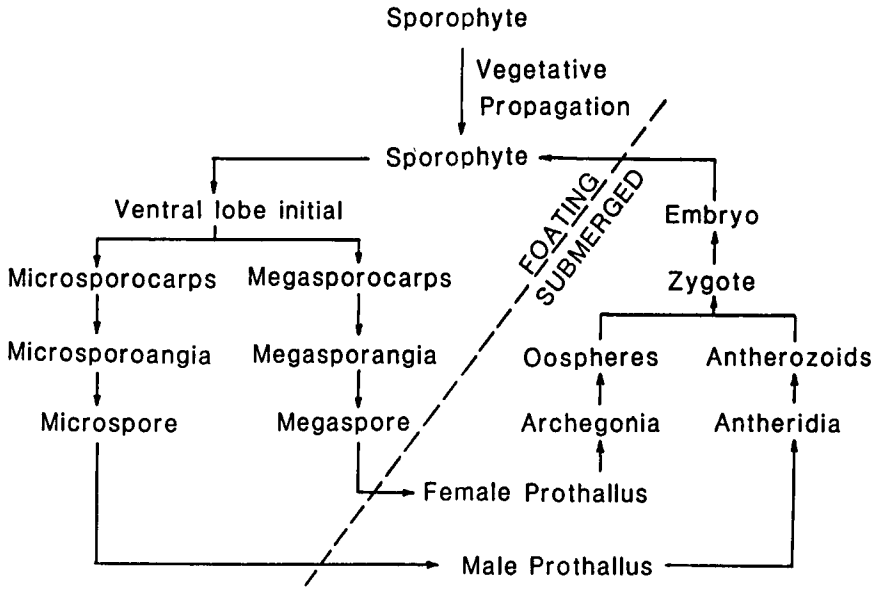


Fig. 1. Life cycle of *Azolla*. *Azolla* is a leptosporangiate heterosporous fern. The plant is a sporophyte, i.e., the diploid generation which produces haploid spores. Sporocarps are borne in pairs on short stalks. The sporocarp pair may comprise two microsporocarps (male), two megasporocarps (female), or one of each. During sporocarp development the megaspore mother cell derived from the ventral lobe initial divides, ultimately producing 32 megaspore nuclei. At this stage either all but one of the nuclei abort, with the survivor giving rise to a megaspore, or they all abort and microsporangial initials arise from basal outgrowths on the stalk of the megasporangium which includes one megaspore and the surrounding megaspore apparatus. Mature microsporocarps may contain anywhere from 8 to 130 stalked microsporangia, each of which may develop 32 or 64 microspores aggregated into three to ten massulae. Each massula consists of a mass of microspores embedded in a mucilaginous matrix originating from the sporangial wall. The mature microsporocarps disintegrate, releasing massulae with glochidia which serve to anchor the massulae to the megaspores. The mature megaspores germinate into female gametophytes, each of which produces one or more archegonia and oospores. The microspores germinate into male prothalli, which differentiate antheridia and release antherozoids. Fertilization takes place either under water or on wet surfaces of gametophytes and results in a zygote which develops into the embryo and the mature sporophyte (Konar & Kapoor, 1974; Lucas & Duckett, 1980; Lumpkin & Plucknett, 1980; Peters & Calvert, 1983).

branch. The sporocarp pair may comprise two microsporocarps (male), two megasporocarps (female), or one of each. A megasporocarp contains a single megasporangium with one megaspore (which includes a food reserve composed of protein bodies, lipid globules, polysaccharide vacuoles, and amyloplasts) and the surrounding megaspore apparatus, which include floats and capture mechanism. Mature microsporocarps may contain anywhere from 8 to 130 stalked microsporangia, each of which may develop 32 or 64 microspores aggregated into three to ten massulae (Moore, 1969). As the endophyte is present throughout the life cycle of the fern,

the production of spores as a possible means of distributing *Azolla* for use as biofertilizer is particularly intriguing for the future (Lumpkin, 1985; Shi & Tang, 1982, 1984; Stewart, 1982).

C. TAXONOMY AND BIOGEOGRAPHY

The genus *Azolla* is usually included with *Salvinia* in the Salviniaceae (Bailey, 1949; Benson, 1957; Black, 1948; Lawrence, 1951; Smith, 1938). Another suggestion is to place *Azolla* in a separate family, the Azollaceae (Eichler, 1965; Konar & Kapoor, 1974; Melchior & Werdermann, 1954; Sculthorpe, 1967). The name *Azolla* implies that the plant dies under dry conditions (azo: to dry, ollyo: to kill) (Lumpkin & Plucknett, 1980; Moore, 1969).

Species demarcation is based primarily upon reproductive structures. According to Hills and Gopal (1967) there are 25 fossil and 6 extant species which are divided into two subgenera, based on the number of megaspore floats (Florschütz, 1949; Moore, 1969; Svenson, 1944). These are the *Euazolla* (three floats), which is currently considered to include the four New World species *A. filiculoides* Lamarck (type species), *A. caroliniana* Willdenow, *A. mexicana* Presl, and *A. microphylla* Kaulfuss, and the *Rhizosperma* (nine floats), which is currently considered to include the two Old World species, *A. pinnata* R. Brown and *A. nilotica* DeCaisne. Lin (1980) suggested that a subspecies of *A. pinnata, A. imbricata* (Roxb.) Nakai is an independent species.

It has recently been found that under scanning electron microscopy (SEM) the species of the subgenus *Euazolla* have rounded nipples on the surfaces of the dorsal lobes, and those in the subgenus *Rhizosperma* have prolate ones (Shi et al., 1984). They also observed with transmission electron microscopy (TEM) that *A. filiculoides* contains more thylakoids in chloroplasts than *A. imbricata* does, and the grana lamellae have more stacks in the former than in the latter (Shi et al., 1984).

Azolla ferns are found in temperate and tropical aquatic ecosystems throughout the world (Lumpkin & Plucknett, 1980; Moore, 1969; Sculthorpe, 1967; Svenson, 1944; Sweet & Hills, 1971).

Azolla filiculoides is widely distributed. It has been reported throughout the Americas from southern South America to Alaska, Hawaii, Australia, New Zealand, England, Ireland, Alsace, Germany, Czechoslovakia, Japan, and China. Prior to marked human influences, it occurred only in southern South America.

A. caroliniana is indigenous to the eastern United States, Caribbean, the West Indies and Mexico, but has also been introduced into eastern Spain, France, Italy, and China.

A. mexicana is found in northern South America through western North America to British Columbia, and east to Illinois.

A. microphylla occurs in western and northern South America, subtropical North America, and the West Indies.

A. pinnata (and/or *A. imbricata*) is widespread in the Eastern Hemisphere. It has been reported from tropical Africa, southern Africa and Madagascar, Australia, New Caledonia, Indonesia, Ceylon, India, Indochina, Japan, and China.

A. nilotica is reported as a large species occurring from the upper reaches of the Nile to the Sudan.

Usually, *Azolla* species have been found in freshwater environments such as ponds, marshes, canals, drainage ditches and, significantly, rice paddies. Since wind and wave action as well as other turbulence causes fragmentation and diminished growth, *Azolla* is not found on large lakes or swiftly moving waters (Ashton, 1974). Although *Azolla* can colonize bodies of water that are nitrogen deficient, their growth can be limited by the availability of other nutrients, especially phosphorus and iron (Olsen, 1972; Singh, 1979; Talley et al., 1977; Watanabe, 1978, 1982, 1984, 1986; Watanabe et al., 1977, 1981).

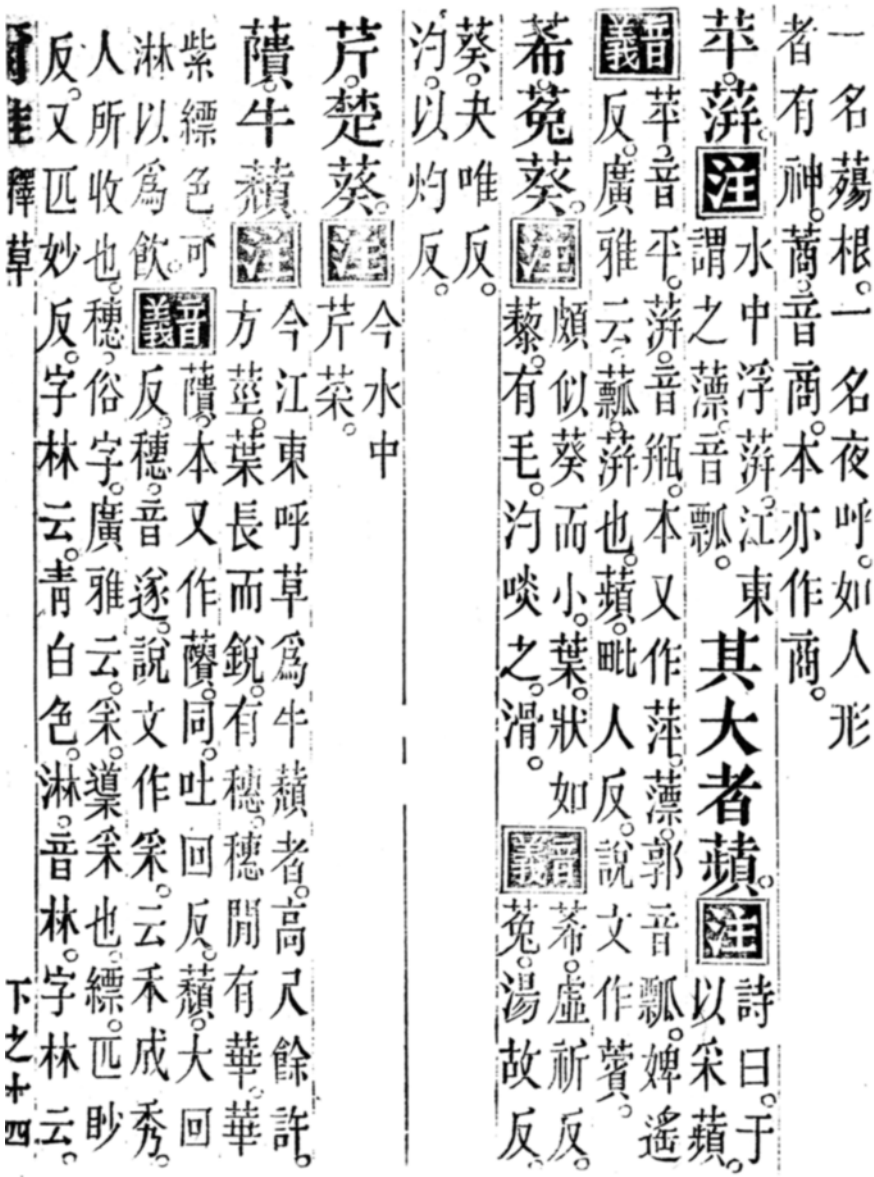
Since the information concerning *Azolla* was last reviewed in this series by Moore (1969), the subject has expanded at an impressive rate, particularly with respect to our understanding of symbiosis and energy metabolism. For a more general perspective on *Azolla,* readers are directed to reviews made by Peters et al. (1982, 1986), Lumpkin and Plucknett (1982), Shi and Tang (1982, 1984), and Watanabe (1982, 1984).

III. Historical Perspective

A. AGRICULTURAL USE

The *Azolla-Anabaena* associations have a long history of use as a green manure for rice and as fodder for poultry and livestock in China and other Far East countries.

The exact period when Chinese people began to use them has not been recorded. The earliest mention of the plant seems to be in the Er Ya (尔 雅), an ancient dictionary that appeared about 2000 years ago (Fig. 2a). Guo Po's (郭 璞) commentary says: "Ping (萍), Piao (蓱): this is a water plant, called also Fu Ping (浮 萍)." (Dr. F. Bray, pers. comm. 1984). During the same period, there were some poems which described the scene where the water plants were picked up, in the earliest collection of Chinese poems "Shijing (诗 经)" ("The Book of Songs"). It was said that the duckweed described was probably *Spirodela polyrhiza* or another member of the Lemnaceae (Lumpkin & Plucknett, 1982). However, although *Azolla* and *S. polyrhiza* grow in the same niche, *Azolla* is usually dominant during the growing season. In 540 A.D. the Chinese book on agricultural techniques written by Jia Si Xue (贾思勰), entitled The Art


一名蕵根。一名夜呼。如人形者。有神。蒿音商。本亦作商。

莘。薍注水中浮薍。江東謂之藻。音瓢。其大者蘋。注詩曰。于以采蘋。

音義 莘音平。薍音紙。本又作萍。藻。郭音瓢。婢遙反。廣雅云。蘞薍也。蘋。毗人反。說文作蓱。

蒿。蒌葵。注頗似葵而小。葉狀如藜。有毛。汋啖之滑。

音義 蒿。虛祈反。蒌。湯故反。

芹。楚葵。注今水中芹菜。

蘋。牛蘋。注今江東呼草爲牛蘋者。高尺餘。許方莖。葉長而銳。有穗。穗開有華。華紫縹色。可淋以爲飲。

音義 蘋。本又作蘱。同。吐回反。蘋。大回反。穗。音遂。說文作采。云禾成秀。人所收也。穗。俗字。廣雅云。采。選采也。縹。匹眇反。又匹妙反。字林云。青白色。淋。音林。字林云。

下之十四

Fig. 2. (A) The earliest mention of *Azolla* in the Er Ya (尔 雅) which is an ancient dictionary that appeared about 2000 years ago. (B) In 540 A.D. the Chinese scientist Jia Si Xue (贾思勰) mentioned Er Ya in the section on applied plant cultivation in his book "Qi Min Yao Shu" (齐民要术) ("The Art of Feeding the People").

吾九疑人也聞嵩岳有石上菖蒲一寸九節可以長生故來採之忽然不見帝謂侍臣曰彼非欲服食者以此喻朕耳乃採菖蒲服之帝服之煩悶乃止與服不止遂以長生

薇

召南詩曰陟彼南山言采其薇詩義疏云薇山菜也莖葉皆如小豆藿可羹亦可生食之今官園種之以供宗廟祭祀也

萍

爾雅曰萍萍也其大者蘋呂氏春秋曰菜之美者崐崙之蘋

石蓴 文之切

爾雅曰潭石衣郭璞曰水蓴也一名石髮江東食之潭葉似鼸而大生水底亦可食

胡荽

爾雅云蔧耳苓耳

廣雅云泉耳也亦云胡泉郭璞曰胡荽也江東呼爲常泉

周南云采采卷耳毛云苓耳也注云胡荽也詩義疏曰苓似胡荽白花細莖蔓而生可臛爲茹滑而少味四月中生子如婦人耳璫或云耳璫草幽州人謂之爵耳

博物志洛中有驅羊入蜀胡葱子著羊毛蜀人取種因名羊負來

of Feeding the People [Qi Min Yao Shu (齐民要术)] put the Ping and Piao mentioned in Er Ya into the part of applied plant cultivation (Fig. 2b). The medicinal properties of *Azolla* were described in the Compendium of Materia Medica [Bencao Gangmu (本草纲目)] written in the 16th century by Li Shi-zhen (李时珍).

Dao and Tran (1979) reported that *Azolla* was used about the 11th century in Vietnam. Separate mythologies place the original sites of cultivation in the district of Wenzhou (温 州), Zhejiang Province, China, and the village of La Van, Thai Binh Province, Vietnam.

A push for expanding the use of *Azolla* began in China in the early 1960's and caught the interest of other countries in the early 1970's. By 1977, *Azolla imbricata* (*A. pinnata* the only species native in China) cultivation was being practiced in Southern China where rice is grown. This species cannot overwinter outside and propagates slowly in early spring. "Perhaps the most significant breakthrough for the improvement of *Azolla* cultivation in China was the importation of a variety of *A. filiculoides*" (Lumpkin & Plucknett, 1982). *A. filiculoides* is an unusually cold-tolerant variety which allowed the cultivation of *Azolla* to expand into Northern and Northeastern China and allowed for the increased production of *Azolla* inoculum at an earlier date in the southern provinces. The introduction of a few plants of *A. filiculoides* from East Germany by the Institute of Botany, Academia Sinica in 1977 was multiplied until they covered 250,000 ha by 1979 (Lumpkin & Plucknett, 1982; Shi et al., 1981; Shi & Tang, 1984). Usually vegetative reproduction is common for *Azolla* but it was observed that the sporogenesis of *A. filiculoides* was more predictable than that of the native *A. imbricata*. "Chinese researchers have led the world in developing procedures for the collection and use of *Azolla* spores, improving methods for the oversummering and overwintering of the sporophyte and in detailing the life cycles of insects which attack *Azolla*" (Lumpkin, 1985).

By 1980, *Azolla* cultivation was being practiced in Thai Binh, Nam Dinh, Hai Duong, and Hung Yen Provinces of Vietnam and in isolated areas in Senegal. In addition, it was being investigated for use by researchers in Bangladesh, Burma, Egypt, India, Indonesia, Malaysia, Nepal, Peru, the Philippines, Thailand, Senegal, Sri Lanka, Hawaii and California in the USA, and by the West African Scientifique et Technique Outre Mer (ORSTOM) in Africa, Brazil, Japan, Italy, and Puerto Rico (Lumpkin & Plucknett, 1982; Silver & Schröder, 1984).

B. HISTORY OF RESEARCH

The history of research on *Azolla* may be divided into three periods since Lamarck established the genus *Azolla* in 1783 after examining spec-

imens brought from Chile (Griffith, 1845). During the first 90 years, the main work was about the classification of *Azolla* species (Mettenius, 1847). Meyen (1836) introduced *Euazolla* to replace the earlier section *Azolla.*

In 1873, the first monograph on *Azolla* was published (Strasburger, 1873). He studied the anatomy of *Azolla* and mentioned *Anabaena azollae* as the symbiont. From then on, the geographic distribution, morphology, cytology, and physiology have been studied (Konar & Kapoor, 1972, 1974; Moore, 1969; Sweet & Hills, 1971). By the early 1970's, it was reported that *Anabaena azollae* was not able to grow apart from the host (Bortels, 1940; Oes, 1913; Shields & Durrell, 1964); attempts to recombine *A. azollae* and endophyte-free *Azolla* had proved unsuccessful (Bortels, 1940; Huneke, 1933; Limberger, 1925; Wildemann, 1934); *Pseudomonas* and *Azotobacter* were always found in the leaf cavity of *Azolla* (Bottomley, 1920); only limited evidence of nitrogen fixation by *Anabaena azollae* was available (Venkataraman, 1962; Vouk & Wellisch, 1931) so that there was no conclusive evidence for *Anabaena azollae* being the actual agent of nitrogen fixation.

Since the early 1970's there has been a world-wide stimulation of research on biological solar energy conversion and biological N_2 fixation and interest in their potential for alleviating the food and energy crisis. This has resulted in numerous works on *Azolla,* which have focused on the green manure, nitrogen fixation, photosynthesis, hydrogen production and symbiosis (Ashton, 1974; Bai et al., 1978, 1979; Becking, 1976, 1978, 1979; Brotonegoro & Abdulkadir, 1976; Hill, 1975, 1977; Holst & Yopp, 1976; Lumpkin & Plucknett, 1980; Newton, 1976; Peters & Mayne, 1974a, 1974b; Pieterse et al., 1977; Shi, 1981; Singh, 1977, 1979; Talley et al., 1977; Tung & Shen, 1981; Yatozawa et al., 1980; Watanabe et al., 1977, 1981). Peters and his coworkers first found simple methods to isolate the symbiotic cyanobacterium and obtained direct evidence that it is the site of N_2 fixation. During the past decade they have published a series of works on the physiology and biochemistry of *Azolla* that has allowed us to better understand the symbiosis in the association (Calvert et al., 1983, 1985; Calvert & Peters, 1981; Kaplan et al., 1986; Kaplan & Peters, 1981; Perkins et al., 1985; Peters, 1975, 1976, 1977, 1978; Peters & Calvert, 1983; Peters & Ito, 1984; Peters & Kaplan, 1981; Peters & Mayne, 1974a, 1974b; Peters et al., 1976, 1977, 1978, 1979, 1980a, 1980b, 1981a, 1981b, 1982, 1985a, 1985b, 1986; Ray et al., 1978, 1979; Toia Jr. et al., 1981, 1985; Tyagi et al., 1980, 1981).

Newton and Herman (1979) have developed a successful method for isolating cyanobacteria from *Azolla* tissue and capable of growth in vitro. Based on such methodological progress, some work on immobilization (Hall et al., 1985; Shi et al., 1987a, 1987b, 1988) and molecular genetics of *A. azollae* (Franche & Cohen-Bazire, 1985; Nierzwicki-Bauer & Hasel-

korn, 1986) has recently appeared. It is implied that a new biotechnological stage of *Azolla* research has begun.

C. RESEARCH IN CHINA

Azolla has been of traditional interest to Chinese botanists and agriculturists. The Chinese began to research the environmental constraints on *Azolla* use in the 1950's, when cultivation was restricted to the use of native *A. imbricata* (*A. pinnata*). Early research focused on the life cycles of insect pests, plant physiology, oversummering and overwintering of the sporophyte and use as a green manure (Institute of Soil and Fertilizer, Zhejing Academy of Agricultural Sciences, 1975; Lumpkin & Plucknett, 1982). "The introduction of the species *Azolla filiculoides* in 1977 is the major factor affecting current research in China" (Lumpkin, 1985). Current research has expanded to include the variation in species and varieties, isolation and culture of the cyanobacterial symbiont, nitrogen fixation, hydrogen metabolism, relationship between fern and symbiont, elucidation of the sexual cycles and use of the spores. China has organized hundreds of people to study both basic and applied areas of interest and they have published about 500 papers over the last 30 years.

IV. Symbiosis

A number of studies have considered the morphology and symbiosis of *Azolla* species (Bonnet, 1957; Demalsy, 1953, 1958; Gunning, 1978; Konar & Kapoor, 1972; Peters & Calvert, 1983; Peters et al., 1980a; Queva, 1910; Rao, 1936; Shi et al., 1984; Sud, 1934; Sweet & Hills, 1971). The electron microscope has been used to observe leaves (Calvert & Peters, 1983; Calvert et al., 1985; Kawamatu, 1965a; Shi et al., 1984), megaspores (Martin, 1976), microsporogenesis (Herd et al., 1985), root caps (Kawamatu, 1962), chloroplasts in root hairs (Kawamatu, 1961, 1962), cortical microtubules (Gunning, 1978, 1980; Gunning et al., 1978a, 1978b, 1978c), the role of transfer cells in the symbiosis (Duckett et al., 1975; Peters, 1976), and *Anabaena azollae* in cavities (Grilli, 1964; Kawamatu, 1965a, 1965b; Lang, 1965; Lang & Whitton, 1973; Shi et al., 1984).

A. MORPHOLOGY OF *AZOLLA*

Sporophytes of *Azolla* consist of multibranched, prostrate, floating stems (rhizomes) that bear deeply bilobed leaves and determinant, adventitious roots (Fig. 3a and b). The extensive branching pattern results in numerous stem apices and a growth habit that ranges from flabellate to polygonal, depending upon the degree and pattern of fragmentation. An abscission

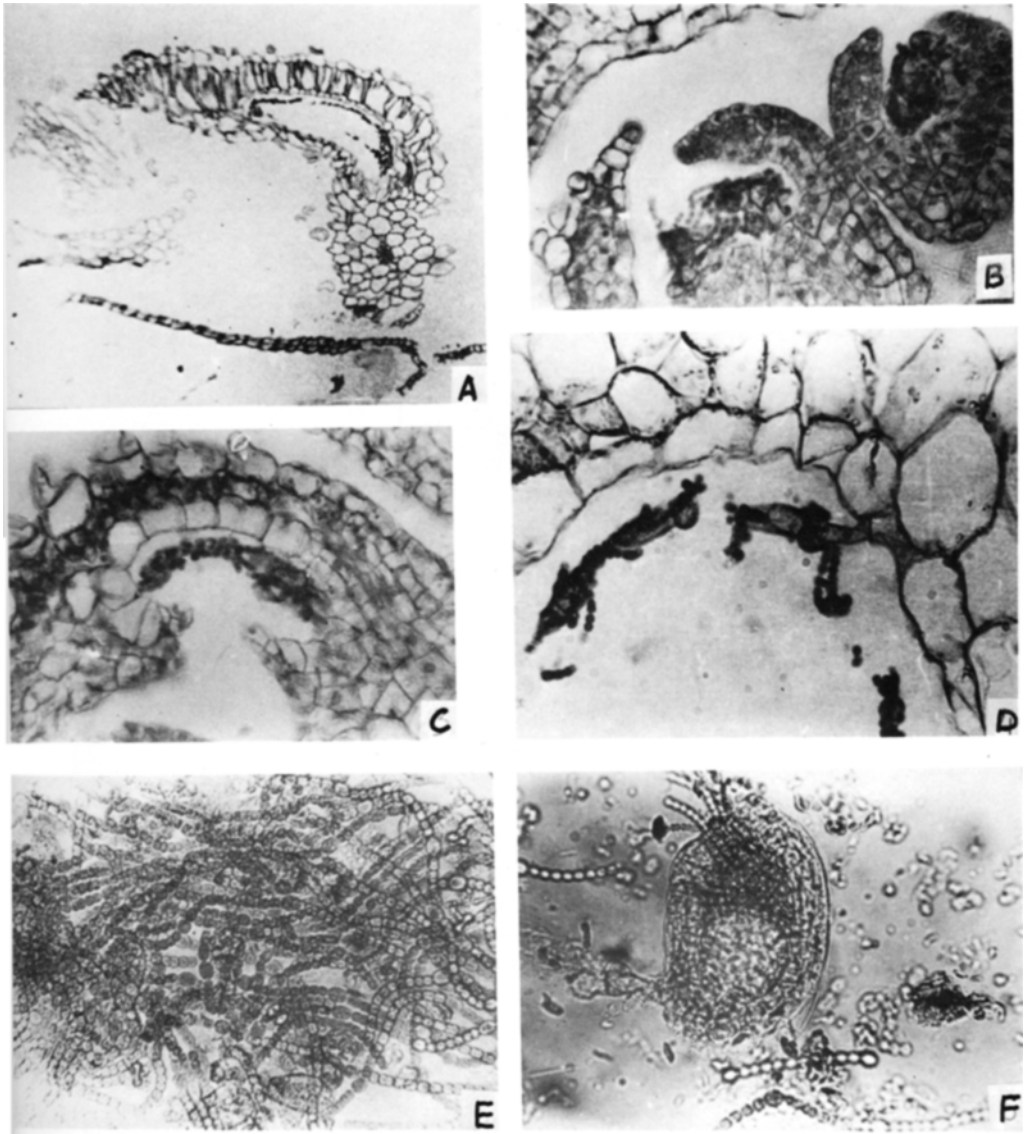


Fig. 3. Micrography of symbiosis between *Azolla* and *Anabaena azollae*: (A) Symbiotic cyanobacterium cells in a mature leaf cavity; (B) Cyanobacterium cells around the leaf primordium; (C) Cyanobacterium cells entering a leaf cavity; (D) Hair cells which come from the fern associated with the cyanobacterium symbiont; (E) Cyanobacterium filaments gathered together and exhibiting a higher frequency of heterocysts than at earlier stages of development; (F) Cyanobacterium filaments appear in the macrospores.

layer at the point of root and branch attachment facilitates vegetative propagation through fragmentation. The diameter of the sporophyte is usually about 1 cm for *A. pinnata* (*A. imbricata*), *A. mexicana, A. microphylla,* and *A. caroliniana,* 5–7 cm for *A. filiculoides,* and *A. nilotica* can grow to 40 cm or more.

Two lateral rows of leaves are borne alternately on the rhizome and may overlap. Each leaf has two lobes of approximately equal size, one dorsal and the other ventral. The thin ventral lobe is nearly colorless and floats on the water surface. The distal half of its blade is only one cell thick. The mature dorsal leaf lobe is aerial and has a clearly defined multilayered mesophyll as well as adaxial and abaxial epidermal tissues. The abaxial epidermis has many stomata. Single-celled papillae are also present on the abaxial epidermis. The leaf margins are entire in all species. Each mature dorsal leaf lobe has an ellipsoid cavity in the proximal half of its lamina. The cavity normally contains the endophyte, *Anabaena azollae* which is associated with each dorsal leaf lobe throughout its development. The implied relatedness of the endophyte in the various *Azolla* species has recently been supported by the report that the symbiotic *Anabaena* from six *Azolla* species share identical and highly specific antigens (Arad et al., 1985; Ladha & Watanabe, 1982, 1984).

B. *ANABAENA AZOLLAE*

Anabaena azollae Strasburger is the only species mentioned in the symbiotic cavity of *Azolla.* Taxonomists place it within the phylum Cyanophyta, order Nostocales, family Nostocaceae. The species has sinuous trichomes (threads) composed of bead-like or barrel-shaped cells without a sheath (Geitler, 1925; Lumpkin & Plucknett, 1980; Shen, 1960; Tilden, 1910). Fjerdingstad (1976) claimed that the cyanobacterium is actually an ecoform of *Anabaena variabilis.* His proposal was based on second-hand information and a specimen of *Azolla* supposedly containing a heterocyst-free cyanobacterium.

As with other heterocystous cyanobacteria, there are three types of cells—vegetative cells, heterocysts, and the akinetes in a trichome of *A. azollae.* Usually spores cannot be observed (Hill, 1977; Prescott, 1951; Tilden, 1910). The average frequency of heterocysts observed by Peters (1975) was 23.1%, with the remainder composed of 60.9% vegetative cells and 16% akinetes (or spores). *A. azollae* akinetes are contained under the developing indusium (cap) of both the microsporocarps and megasporocarps (Bonnet, 1957; Campbell, 1893; Shen, 1960; Smith, 1955; Strasburger, 1873). When an akinete germinates, its contents divide and form a short filament (hormogonium). The spore membrane becomes mucilaginous, swells, and then ruptures releasing its contents (Fritsch, 1904;

Shen, 1960). After various attempts, Shen (1960) found she could induce formation of akinetes by running tap water over *Azolla* fronds. Lang (1965) and Grilli (1964) observed the sequential development of heterocysts from vegetative cells in *A. azollae* under the electron microscope. Lang (1965) noted similarities to heterocyst development in *A. cylindrica.*

Azolla and its endophyte *Anabaena azollae* exhibit a coordinated pattern of differentiation and development (Fig. 4) (Hill, 1975, 1977; Peters & Calvert, 1983; Shi et al., 1984) and there may be a recognition mechanism (Kobiler et al., 1981). Undifferentiated, non-nitrogen-fixing *Anabaena* filaments are associated with the apical meristem of each main and lateral branch of the sporophyte's floating rhizome. Leaf cavities begin as depressions in the adaxial epidermis of the developing dorsal leaf lobes.

An epidermal hair, termed the primary branched hair (PBH) originates in the axil of the forming dorsal lobe. Growth of the PBH is directed toward the apical *Anabaena* colony. Its terminal cells have transfer cell ultrastructure and it is probably metabolically interactive with the *Anabaena* filaments (Fig. 4a). As the developing leaves are displaced from the meristem, *Anabaena* filaments remain associated with the PBH and are thereby partitioned into the forming leaf cavities. Concomitant with the onset of cavity closure, the *Anabaena* cells enlarge and heterocysts are rapidly differentiated with the occurrence of nitrogenase activity; other branched hairs form and numerous simple hairs emerge from the cavity wall. The two branched hairs occupy similar positions along the path of the foliar trace in each cavity. More than 20 simple hairs develop around those portions of a mature cavity adjacent to photosynthetic mesophyll (Calvert & Peters, 1981). In a mature cavity, the *Anabaena* filaments are localized around the periphery of the cavity in close proximity to the epidermal hairs (Shi et al., 1984). A procedure has been developed to isolate hair cells from *A. filiculoides* containing a packet of *Anabaena* (Uheda, 1986).

The routes by which metabolite exchange occurs between the partners are still unresolved. Based upon the distribution, morphology, and transfer cell ultrastructure of the two populations of cavity trichomes (Calvert & Peters, 1981; Duckett et al, 1975; Konar & Kapoor, 1972; Peters & Calvert, 1983; Sun et al., 1984), the cavity hairs may play a central role, with the branched hairs involved in ammonia uptake and/or metabolism (throughout development) and the simple hairs providing a possible conduit for the transfer of sucrose from the mesophyll to the endophyte in mature cavities (Peters et al., 1985a).

The interior surface of a mature leaf cavity is lined with an envelope (Peters, 1976) and covered by a mucilaginous layer of unknown composition in which *A. azollae* filaments, multicellular transfer hairs and a few bacteria are found (Fig. 4b) (Bottomley, 1920; Gregor, 1938; Grilli,

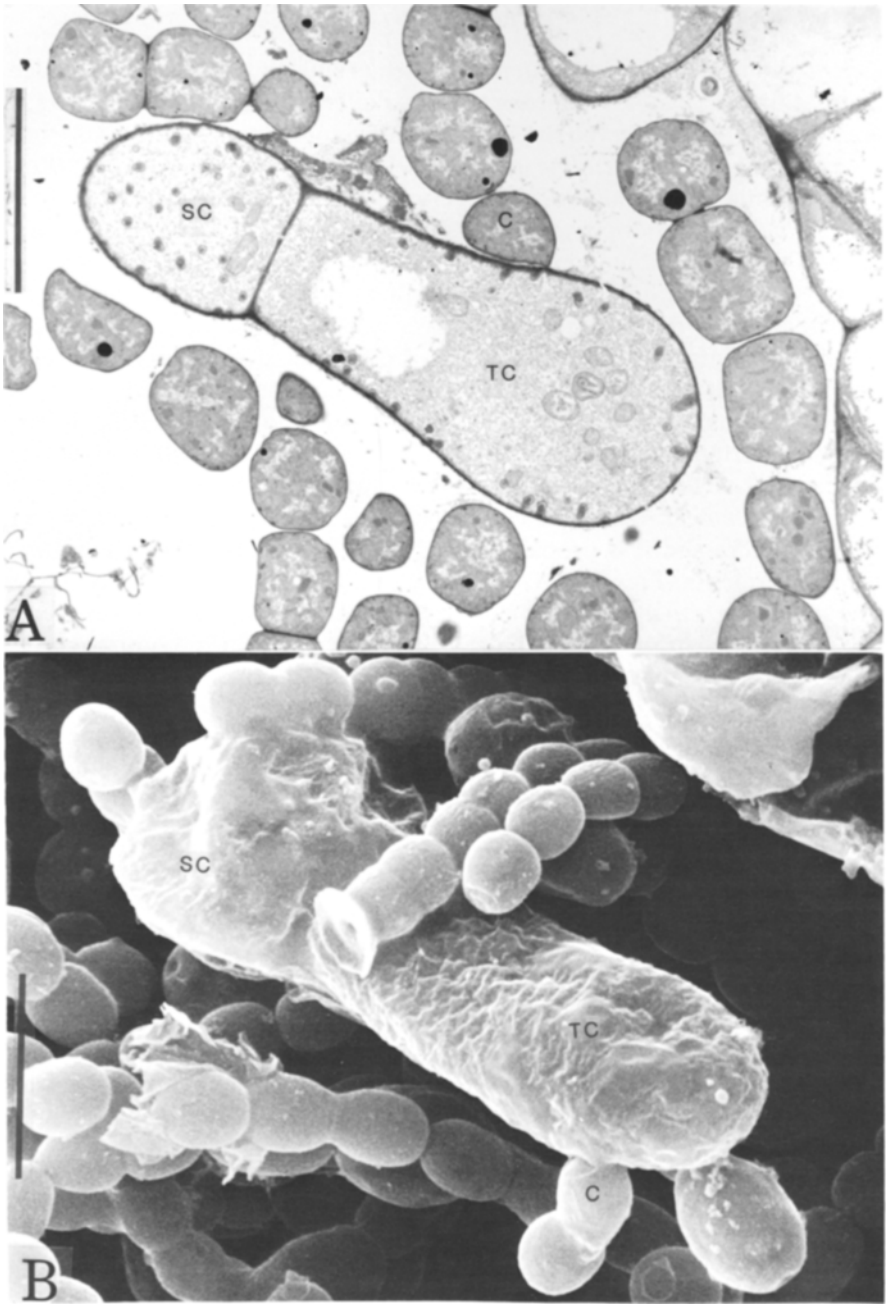


Fig. 4. The connection between a host hair cell and symbiotic *A. azollae* in the leaf cavity. (A) Transmission electron micrograph. The mature simple hair cell consists of a terminal cell (tc) and a stalk cell (sc). Bar = 10 μm; (B) Scanning electron micrograph. The cyanobacterial cells (c) are seen to be adhering to a simple hair cell (h). Bar = 10 μm.

1964; Peters, 1976; Wierienga, 1968). Moore (1969) presumed that the mucilage was secreted by the transfer hair, but Duckett et al. (1975) found that cavities freed of the symbiont did not contain mucilage. They speculated that mucilage normally found in the cavities was probably derived from the symbiont. With foam-immobilized presumptive *A. azollae,* it has also been shown that the mucilage was produced by the cyanobacterium (Robins et al., 1986). Schaede (1947) and Grilli (1964) claimed that liquid fills the whole cavity, but observations by Lumpkin and Plucknett (1980) indicated that the cavity is lined with mucilage and largely filled with gas. Nitrogen compounds of the leaf cavity liquid have been identified (Xu et al., 1983).

A. azollae shares the leaf cavity with small populations of bacteria. Bottomley (1920) mentioned isolating *Pseudomonas* and *Azotobacter* from the cavity. Peters and Mayne (1974a, 1974b) concluded that they were non-nitrogen fixing. Isolated cultures of *A. azollae* were freed of bacteria by either ultraviolet radiation (Venkataraman, 1962) or heat treatment at 47°C for 100 minutes (Wierienga, 1968).

C. CYANOBACTERIUM-FREE *AZOLLA*

Endophyte-free *Azolla* may rarely occur in nature (Fremy, 1930; Hill, 1977; Marsh, 1914), but can be generated by several techniques. Moore (1969) reviewed the early methods claiming to produce cyanobacterium-free *Azolla* fronds. These methods involved growing *Azolla* under conditions of environmental stress, such as cold, low light, and nutrient deficiency (Huneke, 1933; Limburger, 1925). Nickell (1958) used antibiotics for producing cyanobacterium-free *Azolla.* He treated *Azolla* sequentially in penicillin, terramycin, and streptomycin sulfate for one week each until *Azolla* was freed of *A. azollae* and contaminating microorganisms. His method was successfully employed by Johnson et al. (1966), Peters and Mayne (1974a), and Ashton and Walmsley (1976). Hill (1975, 1977) produced endophyte-free *Azolla* by first growing *Azolla* under low light intensity (1250 lux) and then under high light intensity (10,000 lux), a method similar to that reported by Schaede (1947). Pure cultures of *Anabaena*-free *Azolla* were also obtained by surface sterilizing stem apices (Bai et al., 1979; Duckett et al., 1975).

D. ISOLATION OF SYMBIOTIC CYANOBACTERIUM

Two techniques have been developed for isolating *A. azollae* from *Azolla* fronds. One is a gentle pressing method by which the fronds are mildly squashed with a roller, followed by filtering and centrifugation (Peters & Mayne, 1974a). The other is enzymatic digestion by which cyanobacterial packets can be obtained (Peters, 1976).

Numerous authors (Ashton & Walmsley, 1976; Becking, 1976; Huneke, 1933; Shen, 1960; Tuzimura et al., 1957; Venkataraman, 1962; Vouk & Wellisch, 1931; Wierienga, 1968) claimed to have grown *A. azollae* in isolation, but there are some different opinions (Bortels, 1940; Hill, 1975; Lang, 1965; Peters, 1976; Singh, 1977; Walmsley et al., 1973). Newton and Herman (1979) developed a procedure to isolate cyanobacteria from *Azolla*; the method is based upon recovery of cyanobacterial "bundles" from digests of plants and use of this material as a massive inoculum in nitrogen-free media, followed by prolonged incubation in light. Isolated cyanobacteria were found to resemble *Anabaena* sp. morphologically but were capable of heterotrophic growth and had high nitrogenase activity when grown on fructose in the dark. Since then, cultured isolates of *A. azollae* were obtained in several laboratories for comparative studies with the fresh isolates (Gates et al., 1981; Ladha & Watanabe, 1982; Tel-Or et al., 1983).

Although the cultured isolate has exhibited some characteristics different from general free-living cyanobacteria (Newton & Cavin, 1985; Rozen et al., 1986; Shi et al., 1987; Tel-Or & Sandovsky, 1982; Wu et al., 1982), there are still doubts. In addition to the morphological differences (Newton & Herman, 1979), an obvious difference in surface antigenicity appeared to exist between fresh *A. azollae* cells and those obtained after in vitro culturing (Arad et al., 1985; Gates et al., 1981; Ladha & Watanabe, 1982, 1984). Based on the completely different hybridization patterns observed with restriction digests of the cultured cyanobacterial isolate from *A. filiculoides,* Franche and Cohen-Bazire (1985) have recently concluded that this cultured isolate is not closely related to any of the *Euazolla* symbionts. Nierzwicki-Bauer and Haselkorn (1986) have the same opinion on their culture isolated from *A. caroliniana.*

Identification of strains as effective cyanobacterial symbionts of *Azolla* will require their reintroduction into cyanobacteria-free plant material (Koch's postulates). A successful recombination of *Azolla* freed of its symbiont with free-living isolates is at present restricted to a single report (Liu et al., 1984). Thus, the cultured isolate might better be called a presumptive *Anabaena azollae* (G. A. Peters, pers. comm., 1986).

V. Energy Metabolism

A. PHOTOSYNTHESIS

Both the eukaryotic *Azolla* and the prokaryotic *Anabaena* are photosynthetic organisms. Since *Azolla* chloroplasts contain chlorophylls a and b as well as carotenoids, while the *Anabaena* filaments contain chlorophyll a, the phycobiliproteins—phycoerythrocyanin (λ max 570 nm, shoulder

590 nm), phycocyanin (λ max 610 nm), and allophycocyanin (λ max 647 nm, shoulder 620 nm)—and carotenoids (Becking & Donze, 1981; Kaplan et al., 1986; Peters & Mayne, 1974a; Shi & Tang, 1982; Shi et al., 1983; Tyagi et al., 1980, 1981), the light-harvesting pigments of the partners are complementary. In the *A. caroliniana-Anabaena* association the endophyte accounts for 10–20% of the association's total chlorophyll and about 16% of its total protein, with phycobiliproteins accounting for 4–10% of the endophyte's protein (Peters, 1978; Peters & Mayne, 1974a; Ray et al., 1978). In addition to photosynthetic pigments, *Azolla* may also contain anthocyanins, predominantly luteolinin-5-glucoside, with lesser amounts of apigeninidin glucoside (Holst, 1977; Pieterse et al., 1977). Anthocyanin formation can be triggered by a variety of environmental factors, e.g., temperature, pH, nutrition. Their production can cause the *Azolla* to take on a variety of reddish hues which is the origin for the Chinese name of *Azolla* "Man Jiang Hong" (满江红), that means "whole river is red."

The net photosynthetic rates have been measured under various growing conditions (Shi, 1981; Vu Van Vu et al., 1986). The photosynthetic light saturation point is about 6000 lux for *A. imbricata* and *A. filiculoides* in spring, and about 8000 lux for *A. imbricata* and about 14,000 lux for *A. filiculoides* in summer. The light compensation point is in the range of 500–1000 lux. For *A. imbricata,* the optimum temperature for photosynthesis is 25–32°C for green plants and 18–32°C for reddened plants. In the range of 5–45°C a net photosynthetic rate may be detected. Optimum pH of the water medium for photosynthesis of green plants is 6 and 5.5 for reddened plants. In the range of pH 4.5–10.5, net photosynthetic rates occur. Under violet plastic film photosynthesis is better than under yellow, green, blue, and red films. The net photosynthesis of the association is twice as high as the cyanobacteria-free *Azolla* (Shi & Tang, 1984). In fact, the net photosynthesis of *Azolla* is very high, about 350–450 mg CO_2/g dry wt·hour. Converting this into a leaf area basis (assuming 25,000 g fresh wt/m^2, and 6% dry wt to fresh wt), the net photosynthesis is about 220–290 mg CO_2/dm^2·hour which is higher than most C_4 plants (Shi, 1981; Shi & Tang, 1984).

The association and individual partners exhibit Calvin cycle (C_3) intermediates of CO_2 fixation (Ray et al., 1979). Sucrose is a primary fixation product in the *Azolla,* but does not occur as a ^{14}C-labeled reaction product in the endophyte. As with other C_3 plants, the association and endophyte-free *Azolla* exhibit an O_2-dependent CO_2 compensation point and photosynthesis is inhibited by atmospheric O_2. Rates of CO_2 fixation in air are about 40% less than those at 2% O_2 (Ray et al., 1979). The aerobic CO_2 compensation point is about 30–40 ppm CO_2 (Shi et al., 1981).

Photosynthesis by the endophyte is not inhibited by atmospheric O_2 and the CO_2 compensation point is about 40 ppm CO_2 at both 20% and 2% O_2 (Ray et al., 1979).

As with other cyanobacteria, the relative quantum yield for photosynthesis of the endophyte is highest between 580 and 640 nm, the region of phycobilin absorption. Action spectra for photosynthesis in the association and endophyte-free *Azolla* are very similar to one another and to other green plants, with the maximum quantum yield occurring between 650 and 670 nm (Ray et al., 1979).

Fern chloroplasts and *Anabaena azollae* obtained from density gradient centrifugation were assayed utilizing the diphenyl carbazide-DCIP (DCMU-sensitive portion) assay for photosystem II and $NADP^+$ reduction and using ascorbate-DCIP H_2 assay for photosystem I (Peters & Mayne, 1974a). In the cyanobacterial fraction, both photosystems are repressed when compared to free-living *Anabaena cylindrica,* but the relative ratio of PSI to PSII may be appreciably greater in *Anabaena azollae. Azolla* chloroplasts were generally comparable to spinach chloroplasts (Peters & Mayne, 1974a).

Fluorescence emission spectra at room temperature show that excitation transfer between chlorophyll a and phycobilin in the symbiotic cyanobacterium seems not as efficient as in the free-living cells (Shi et al., 1983). In the presence of DCMU (5×10^{-5} M) the related fluorescence yield of chlorophyll a and of phycobilin in the symbiont is negligibly enhanced, and photosynthetic oxygen evolution in the symbiont is very low (Shi et al., 1983; Shi & Tang, 1984).

B. NITROGEN FIXATION AND HYDROGEN METABOLISM

The internal source of fixed nitrogen gives *Azolla* a competitive advantage over other floating hydrophytes in many environments. Nitrogen fixation combined with a high growth rate (up to a doubling time of 2 days) can enable *Azolla* to accumulate more than 10 kg of nitrogen/ha/day under good conditions (this can be obtained from an *Azolla* biomass of 25 tonnes fresh wt/day) (Lumpkin, 1985). This is under ideal conditions. Talley et al. (1977) reported a daily fixation rate of 1.2 kg N/ha.

As with free-living heterocystous cyanobacteria, the symbiotic *Anabaena* is able to reduce N_2 under an air atmosphere and the nitrogenase is assumed to be localized in the heterocysts. The absence of CO_2 fixation and the rapid reduction of triphenyltetrazolium chloride (TTC) to red formazan in heterocysts of the symbiont support this concept (Peters, 1975). To some extent the *Azolla* fern is subject to the same limitations as free-living cyanobacteria but it has the advantage in being readily distinguishable by the farmer, can fix N_2 in the presence of combined

nitrogen, and in general is more tolerant of low pH, salinity, etc. than are free-living cyanobacteria (Ashton & Walmsley, 1976; Aziz & Watanabe, 1983; Brill, 1977; de Fiore, 1984; Florenzano et al., 1980; Hermelink & Kramer, 1986; Kumarasinghe et al., 1986; Lumpkin & Bartholomew, 1986; Okroronkwo & Van Hove, 1986; Subba Rao, 1982; Swaminathan, 1984; Tung & Shen, 1985; Vincenzini et al., 1985; Watanabe & Roger, 1984).

Nitrogenase activity requires a source of ATP and reductant. While N_2 is the natural substrate, nitrogenase is capable of reducing a number of other substrates, the most notable being the reduction of acetylene to ethylene and the ATP-dependent reduction of protons to H_2 in the absence of any other reducible substrate. Under anaerobic dark conditions the reduction of all substrates is negligible. Dark aerobic reductions occur but they are dependent upon the endogenous supply of reductant accumulated during prior photosynthesis; the rates are 40–60% of those obtained aerobically in the light. The reduction of all substrates is maximal under anaerobic or microaerobic conditions in the light (Becking, 1976; Peters & Mayne, 1974b; Shi et al., 1981).

It used to be said that nitrogenase requires 2 electrons to reduce C_2H_2 to C_2H_4 and 6 electrons to reduce N_2 to $2NH_3$. Theoretically, a conversion ratio of $3C_2H_2$ reduced per N_2 fixed should exist (Becking, 1976; Brotonegoro & Abdulkadir, 1976). However, an atmosphere with C_2H_2 as a substrate suppresses H_2 production, while an atmosphere with N_2 as a substrate continues to use electrons to produce hydrogen. Peters et al. (1977) compared the partial pressure of 0.1 atmosphere C_2H_2 (95% inhibition of H_2 production) and various partial pressures of a mixture of $^{14}N_2$ and $^{15}N_2$, with H_2 production. They concluded that the conversion factor for C_2H_2/N_2 is actually between 1.6 and 2.0 for the association and 2.5 and 3.0 for the symbiont. Watanabe et al. (1977) found C_2H_2/N_2 conversion ratios for *A. pinnata* of 3.4, 1.6, and 2.4 after 14, 19, and 22 days of growth, respectively. Recently, the stoichiometry of $1H_2$ evolved/$1N_2$ fixed suggests that H_2 evolution is an inherent property of the N_2 fixation reaction of nitrogenase and allows us to consider the reaction needs 8 electrons, 2 of them being used to reduce $2H^+$ to H_2, and 6 of them used to reduce $1N_2$ to $2NH_3$ (Simpson, 1987). This could explain why C_2H_2/N_2 conversion ratios are greater than 3.

Considering that the cyanobacterial portion of the total plant nitrogen is about 19–17%, Becking (1976) estimated that the nitrogenase activity (C_2H_2 reduction) of the symbiont is 6–10 times higher than the activity of the association and 12–20 times higher than the activity of free-living cyanobacteria.

The *Azolla* association is capable of significant light-dependent, nitrogenase-catalyzed H_2 evolution (Newton, 1976; Peters et al., 1976; Shi

et al., 1981). The absence of a differential effect of m-chlorocarbonyl cyanide phenylhydrazone on H_2 production and C_2H_2 reduction, coupled with the parallel inhibition of both processes by DCMU implies that the production of H_2 is nitrogenase-catalyzed and ATP-dependent (Holst & Yopp, 1976; Peters et al., 1976). The highest rates of H_2 production occur under 0.1–0.15 atm C_2H_2 and 0.01–0.02 atm CO (Peters et al., 1976, 1977; Shi et al., 1981). These observations indicate unidirectional hydrogenase activity in the symbiont (Peters et al., 1977; Ruschel et al., 1987; Shi et al., 1981; Smith et al., 1976). Since hydrogenase oxidizes the H_2 produced by nitrogenase, recycling electrons and ATP into the system, the rates of H_2 production under argon are always less than the rates of acetylene reduction (Peters et al., 1977; Shi & Tang, 1984).

Photosynthesis is the ultimate source of all the ATP and reductant required for nitrogenase activity. Diminished rates under aerobic dark conditions versus those obtained under aerobic light conditions with DCMU imply that dark, respiratory-driven nitrogenase activity may be ATP limited (Peters, 1975, 1976). Simultaneous measurements of photosynthesis, respiration and C_2H_2 reduction in *A. imbricata* demonstrated the immediate dependence of nitrogenase on photosynthetically captured radiation for energy but an indirect dependence on CO_2 fixation (Shi et al., 1981). The strong interaction between photosynthesis and N_2 fixation has also been demonstrated by determining the action spectra for nitrogenase-catalyzed C_2H_2 reduction in the association and in the isolated endophyte (Tyagi et al., 1981). In both of these studies, the relative rates 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; the heterocysts of the endophyte were found to retain in the range of 21–36% (Shi et al., 1987b).

C. AMMONIA EXCRETION AND ASSIMILATION

The isolated symbiont not only fixes nitrogen, but also excretes ammonia (Ashton & Walmsley, 1976; Peters, 1976) and continues to excrete ammonia in an environment with ammonium chloride concentrations as high as 5 mM (Peters, 1975). There are three ammonia-assimilating enzymes. The enzyme glutamine synthetase (GS, EC 6.3.1.2) catalyzes the formation of glutamine (glu) from glutamate (gln). The enzyme glutamate synthase (GOGAT, EC 1.4.7.1) carries out the reductive amination of α-ketoglutarate. The glutamate dehydrogenase (GDH, EC 1.3.1.3) reaction provides the means of reversibly incorporating ammonia into glutamic acid. Both the symbiont association and endophyte exhibit glutamine synthetase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH) activities (Orr & Haselkorn, 1982; Ray et al., 1978; Stewart et al., 1980). Although both partners must be considered capable of as-

similating ammonia, the *Azolla* was estimated to account for about 90% of the association's total GS activity and 80% of its total GDH activity (Ray et al., 1978). The results of the kinetics of incorporation of exogenous $^{13}NH_4^+$ into glutamine and glutamate and of using the glutamine synthetase inhibitor, methionine sulfoximine (MSX), the glutamate synthase inhibitor, diazo-oxonorleucine (DON) and increasing the ammonium concentration to greater than 1 mM, provided evidence for assimilation primarily by the glutamine synthetase-glutamate synthase pathway in *Anabaena azollae* (Meeks et al., 1985).

When exposed to $^{15}N_2$ the isolated symbiont incorporates only 5% of the fixed $^{15}N_2$ into an organic fraction, while releasing up to 35% of the N_2 it fixes into the incubation medium as ammonium (Peters et al., 1980b). Incubation of such *Anabaena* preparations for 10 minutes with $[^{13}N]N_2$ resulted in the formation of four radioactive compounds; ammonium, glutamine, glutamate, and alanine. Ammonium accounted for 66% of the total radioactivity recovered and 58% of the ammonium was in an extracellular fraction. Since essentially no extracellular ^{13}N-labeled organic compounds were found, it appears that ammonium is the compound most probably made available to *Azolla* in the absence of a combined nitrogen source (Meeks et al., 1985). Cyanobacterial symbionts generally have low or undetectable levels of glutamine synthetase (Haselkorn, 1978; Stewart, 1977) and low GS levels have been postulated as a biochemical mechanism explaining ammonia excretion (Stewart, 1977). It has been suggested (Haselkorn, 1978; Rai et al., 1986; Stewart, 1977) that the host plants might produce effector substances which modify the endophyte's ammonia assimilating pathways by inhibiting its GS activity or synthesis. Appreciable levels of GDH were found in the 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, GDH could conceivably provide a regulatory role, enabling it effectively to reassimilate released ammonia at high intracavity ammonia concentrations.

GS is thought to be the principal ammonia assimilating enzyme in the host, especially in transfer hairs (Peters, 1977). Rhodes and Stewart (1974) have developed a procedure for the in vivo determination of GS activity by freezing *Azolla* with liquid nitrogen to render the cells permeable. They found GS activity as high as 0.78 mol/minute·g fresh weight.

D. DEVELOPMENT

As mentioned above, *Azolla-Anabaena* associations have a synchronous development of partners, while other plant-cyanobacterial symbioses do not necessarily exhibit a comparable developmental gradient (Rodgers

& Stewart, 1977; Silvester, 1976; Stewart and Rodgers, 1977). The developmental profile was originally described by Hill (1975).

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 (Calvert & Peters, 1981; Kaplan & Peters, 1981; Peters et al., 1980). Shi et al. (1981) reported that a determination of C_2H_2 reduction activity as a function of leaf age established a developmental gradient in both *A. imbricata* and *A. filiculoides*. In both species activity is negligible in the apex, increases markedly in progressively older leaves, then plateaus and decreases as leaves senesce. H_2 production has another pattern and implicates the occurrence of an uptake hydrogenase.

The absence of nitrogenase activity, as determined with C_2H_2 reduction, in *Anabaena* filaments associated with the plant apex implies that N_2 fixed by the *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. Kaplan and Peters (1981) have demonstrated the transportation using a pulse-chase approach with $^{15}N_2$. They also reported that whereas the nitrogen content and dry matter decreased with increasing leaf age, the carbon : nitrogen ratio increased. 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.

Ray et al. (1978) suggested that the endophyte's GS activity might be associated primarily with the undifferentiated filaments in the apical portion of the stem. Subsequently, Haselkorn et al. (1980) employed an antibody against the purified GS from *Anabaena* 7120. They found that the antigen levels of the endophyte were only 5–10% of those observed in a free-living isolate and that the antigen concentration was greatest in the endophyte associated with younger leaves. Since inhibitors of GS activity are known to moderately increase heterocyst frequencies in free-living cyanobacteria (Ladha et al., 1978; Stewart and Rowell, 1986), there is reason to suspect a gradient in the endophyte's GS, decreasing in parallel with the differentiation of heterocysts and epidermal hairs in the leaf cavities.

The relative contribution of the individual partners to the association's total photosynthetic capability and the extent of interaction in fern-endophyte carbon metabolism are largely unknown. A possible correlation between high heterocyst frequency and an exogenous carbon source is suggested by several observations. In most other plant-cyanobacterial symbioses, in which the endophyte simply exhibits a high heterocyst frequency with no developmental profile, the endophyte loses its capa-

bility to fix CO_2 and becomes dependent upon the plant for a source of fixed carbon (Stewart, 1980). In lichens where the cyanobacterium is the only phycobiont, heterocyst frequencies are comparable to or slightly less than those occurring in the free-living cyanobacterium, that is, 4%. However, in a tripartite association between the same two organisms (lichen and cyanobacterium) and a green alga, the cyanobacterium exhibits heterocyst frequencies in the range of 10–30% (Millbank, 1974). Thus, in the *Azolla-Anabaena* association, Peters and Calvert (1983) postulated a transition from photoautotrophic metabolism in generative filaments to a photoheterotrophic or mixotrophic mode of metabolism with increasing differentiation of heterocysts.

Based on the contribution of the endophyte's chlorophyll to the total chlorophyll of the leaf it occupies, the quantitative relationship of the endophyte and leaf biomass remains relatively constant throughout the developmental profile (Peters et al., 1980a). In contrast, total phycobiliprotein content/g fresh weight increases with leaf age and therefore with greater amounts of the endophyte and an increasing proportion of heterocysts. However, the relative amounts of phycoerythrocyanin, phycocyanin, and allophycocyanin remain unchanged. Moreover, no obvious differences have been detected in the complement of phycobiliproteins from vegetative cell and heterocyst preparations (Kaplan & Peters, 1981).

In fact, the evolutionary development of these *Azolla-Anabaena* associations has provided an efficient model of biological solar energy conversion (Shi & Tang, 1984; Tang, 1979). In the evolution of photoautotrophic organisms not may species have evolved which can carry out both oxygenic photosynthesis and nitrogen fixation (and/or hydrogen metabolism). Photosynthesis makes available the ATP and reductants necessary for nitrogen fixation and hydrogen production, but photosynthetic oxygen evolution tends to inhibit the activities of nitrogenase and hydrogenase. Thus it is difficult for photosynthesis and nitrogen fixation (and/or hydrogen evolution) to be in progress effectively at the same time and in the same space.

In order for N_2-fixing photosynthetic organisms to exist and develop in nature, they must have some mechanisms for integrating these energy metabolisms which both promote and inhibit each other. To date, three types of integration in cyanobacteria have been suggested (Shi & Tang, 1984; Tang et al., 1981): the integration by temporal separation in unicellular or non-heterocystous filamentous cyanobacteria; the integration by special separation in heterocystous filamentous cyanobacteria, and the cooperative integration in the plant-cyanobacteria symbiotic systems. Compared with the other two integrations the cooperative integration in symbiosis of *Azolla-Anabaena* association has the highest activities of photosynthesis and nitrogen fixation. This compatibility is dependent

upon their unique host-symbiont interactions and structure-function relationships, and may be useful for biotechnological application (Shi & Hall, 1988; Shi et al., 1987b; Shi & Tang, 1984).

VI. Concluding Remarks

The *Azolla-Anabaena* association is to date the only plant-cyanobacteria symbiosis which has applied significance. It has been used as green manure, forage, and medicine in China for centuries. The applied significance of the *Azolla-Anabaena* association is due to the efficiency of its biological solar energy conversion through an energy metabolism which includes photosynthesis, nitrogen fixation, and hydrogen metabolism. The efficiency of energy metabolism in the *Azolla-Anabaena* association is related to its symbiosis, structure, and compatibility between photosynthesis and nitrogen fixation (and/or hydrogen metabolism). The energy metabolism and structure of the *Azolla-Anabaena* association present a unique model for biotechnology.

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