THE BOTANICAL REVIEW

VOL. 63 JANUARY–MARCH 1997 NO. 1

Azolla: A Review of Its Biology and Utilization

GREGORY M. WAGNER

Department of Zoology and Marine Biology
University of Dar es Salaam
P.O. Box 35064
Dar es Salaam
Tanzania

I. Abstract

The *Azolla–Anabaena* symbiosis is outstanding due to its high productivity combined with its ability to fix nitrogen at high rates. Because of this, in recent decades, countless studies have been conducted on this association, but with insufficient synthesis and coordination. This paper, therefore, attempts to review and synthesize past and recent findings concerning the biology and utilization of *Azolla* in hopes that this will facilitate increased future collaborative research on this "green gold mine." It reviews the taxonomy,

Copies of this issue [63(1)] may be purchased from the Scientific Publications Department, The New York Botanical Garden, Bronx, NY 10458-5125 USA. Please inquire as to prices.

The Botanical Review 63(1): 1–26, January–March 1997

distribution, morphology, physiology, and reproduction of *Azolla* as well as new developments in its manifold uses.

Because of the growing concern about conservation of the environment and the need for deploying renewable, sustainable resources; the application of *Azolla* as a biofertilizer on agricultural crops, in order to provide a natural source of the crucial nutrient nitrogen, can be very beneficial to the future of our planet. Besides the environmental appropriateness of the use of *Azolla*, for multitudes of farmers in many parts of the world who cannot afford chemical fertilizers, *Azolla* application can enhance their economic status, increasing yields while minimizing costs. Due to the fact that rice paddy fields form an ideal environment for *Azolla*, one of its most suitable applications is on rice.

Besides its utilization as a biofertilizer on a variety of crops, *Azolla* can be used as an animal feed, a human food, a medicine, and a water purifier. It may also be used for the production of hydrogen fuel, the production of biogas, the control of weeds, the control of mosquitoes, and the reduction of ammonia volatilization which accompanies the application of chemical nitrogen fertilizer.

Résumé

Une haute productivité associée avec une capacité à fixer l'azote atmosphérique démontre l'importance de la symbiose entre *Azolla* et *Anabaena*. Ce fait fut responsable des multiples études conduites et de l'intérêt porté à cette association durant les dernières décénnies. Mais il semblerait qu'une synthèse ainsi qu'une coordination entre ces différentes entreprises soient manquantes. L'article présenté ici tente de rassembler et synthetiser les résultats accumulés sur la biologie et l'utilsation possible d'*Azolla*, tout en espérant ainsi faciliter la collaboration future entre chercheurs investigant cette "mine d'or vert." La taxonomie, la distribution, la morphologie, la physiologie et la reproduction d'*Azolla* seront couverts, ainsi que ses potentiels multiples usages.

De plus en plus, la conservation de l'environnement et le besoin d'employer des ressources renouvelables de manière à assurer leur usage continu, sont des priorités importantes. L'utilisation d'*Azolla* en tant qu'engrais vert pour l'agriculture, dans le but de fournir de l'azote, élément crucial du cycle nutritionel, pourrait offrir une alternative bénéfique pour le future de notre planète. *Azolla* peut être utilisée par des agriculteurs n'ayant pas les moyens d'employer des engrais chimiques, un peu partout dans le monds, de manière responsable pour le bien de l'environnement. Son application ameliorerait leurs revenus de part son impact sur l'accroissement des rendements tout en minimalisant le coût de production. Un des exemples les plus convaincant est son utilisation pour la culture du riz, etant donné que les champs de riz représentent un environnement idéal pour *Azolla*.

Outre son emploi en tant qu'engrais vert pour différentes productions agricoles, *Azolla* peut aussi très bien être utilisée en tant que nourriture pour les productions animalières, pour la consommation humaine, comme composant pharmaceutique, ou être utilisée dans un processus de purification de l'eau. Son emploi peut aussi inclure la production d'hydrogène en tant que combustible, la production de biogaz, le controle de plantes non desirées, le controle des moustiques, ou participer à la réduction d'émission d'azote lors de l'épandage d'engrais chimiques.

II. Introduction

Azolla–Anabaena is a symbiotic complex in which the endophytic blue-green alga *Anabaena azollae* Strasburger lives within the leaf cavities of the water fern *Azolla* Lam. The endosymbiont, which is nitrogen-fixing, provides sufficient nitrogen for both itself and its host (Peters, 1978). The fern, on the other hand, provides a protected environment for the alga and also supplies it with a fixed carbon source (Peters, 1976; Van Hove, 1989).

There is a wealth of literature on the *Azolla–Anabaena* association. In the 1970s and 1980s, besides the publishing of a vast number of research papers, there were several extensive reviews written on *Azolla* and its uses (Peters, 1977; Lumpkin & Plucknett, 1980, 1982; Watanabe, 1982; Van Hove, 1989). Shi and Hall (1988) gave an historical perspective on *Azolla* and reviewed the nature of its symbiosis and energy metabolism. Numerous research papers have also been written in the 1990s. However, there has been no recent review synthesizing new findings on the biology of *Azolla* and new developments in its manifold uses.

Of the many uses of *Azolla* described in this paper, the most outstanding is its application as a biofertilizer in agricultural production due to its ability to fix nitrogen at high rates. Because of the growing and highly justified concern about conservation of the environment and the need for deploying renewable, sustainable resources in every avenue of man's endeavors, one of the most relevant of which is agriculture, applying *Azolla* to crops as a natural source of the crucial nutrient nitrogen can be very beneficial to the future of our planet. Besides the environmental appropriateness of the use of *Azolla*, for multitudes of farmers in many parts of the world who cannot afford chemical fertilizers, *Azolla* application can enhance their economic status, increasing yields while minimizing costs.

However, the best techniques of application need to be determined—often they vary from one environmental situation to another—and such techniques need to be conveyed in an effective way to the users, i.e., the farmers. Despite the vast number of investigations that have been conducted over the past decades, further research is required in order to capitalize on this important natural resource, particularly research springing from a synthesis of knowledge and having a collaborative approach.

This paper, therefore, attempts to review and synthesize past and recent findings concerning the biology and utilization of *Azolla–Anabaena* in hopes that this will facilitate future collaborative research on this "green gold mine."

III. Taxonomy

The genus *Azolla* Lam. (established by Lamarck in 1783) belongs to the Salviniales. Previously, the most widely accepted view was that *Azolla* belonged to the family Salviniaceae and consisted of two subgenera and six living species (Lumpkin & Plucknett, 1980). The subgenus *Euazolla*, characterized by three megaspore floats and septate glochidia, included four species: *A. filiculoides* Lam., *A. caroliniana* Willd., *A. microphylla* Kaulf., and *A. mexicana* Presl. The subgenus *Rhizosperma*, characterized by nine megaspore floats, included two species: *A. pinnata* R. Br., with simple glochidia, and *A. nilotica* Decne., with no glochidia.

Tan et al. (1986) advocated that *Azolla* should not be placed in the same family as the genus *Salvania* but, rather, should be placed in a separate family, the Azollaceae. They recognized the same subgenera (they called them sections), as did earlier taxonomists, stating that sect. *Rhizosperma* is characterized by the presence of papillae all over the vegetative body, the presence of nine megaspore floats, and the presence of simple glochidia; whereas sect. *Azolla* (or *Euazolla*) is characterized by the possession of papillae only on the leaves and some branches, the presence of three megaspore floats, and the presence of well-developed, septate glochidia with terminal anchor-like structures. They recognized the same six species within these sections as did Lumpkin and Plucknett (1980). Although another species, *A. rubra* R. Br., has been added by some taxonomists to sect. *Azolla*, they preferred to regard it as a variety of *A. filiculoides*. A new characteristic they found useful in taxonomy was the type of float morphology such as the smooth type and the "punctured" type.

There have been some recent developments in the taxonomy of *Azolla*. Saunders and Fowler (1992) revised the taxonomy of sect. *Rhizosperma* based on multivariate statistical analyses of ultrastructural, gross morphological, and anatomical characteristics. They more clearly delimited the characteristics of *A. pinnata* and *A. nilotica*. They named and described three distinct subspecies of *A. pinnata*, namely, subsp. *pinnata*, subsp. *asiatica* subsp. nov., and subsp. *africana* stat.

Saunders and Fowler (1993) suggest major revision of the supraspecific taxonomy of gen. *Azolla* on the basis of a review of published morphological data and their own studies involving cytological and cladistic analyses. They, like Tan et al. (1986), suggest that it is more appropriate to place *Azolla* in the monotypic family Azollaceae. They propose that both subgenera and sections should be used in the supraspecific taxonomy of *Azolla*, with subg. *Azolla* being divided into two sections. Section *Azolla* should include five species (*A. filiculoides* Lam., *A. rubra* R. Br., *A. mexicana* Presl, *A. caroliniana* auct. non Willd., and *A. microphylla* auct. non Kaulf.). Section *Rhizosperma* should contain only *A. pinnata* R. Br. Moreover, they propose that *A. nilotica* Decne. ex Mett. be placed in a new subgenus, *Tetrasporocarpia*. They justify this separation of *A. nilotica* into its own subgenus on the bases that it has the unique habit of producing sporocarps in fours, that it has a chromosome number of $2n = 52$ (whereas *A. pinnata* has $2n = 44$ like the species of sect. *Azolla*), and that it is evolutionarily more distant from the species of sect. *Azolla* than is *A. pinnata*.

IV. Distribution

Azolla occurs in freshwater habitats in tropical, subtropical, and warm-temperate regions throughout the world. Prior to intervention by man, *A. caroliniana* was distributed in eastern North America and the Caribbean, *A. filiculoides* in the Americas from southern South America through western North America to Alaska, *A. microphylla* in tropical and subtropical America, *A. mexicana* in the Americas from northern South America through western North America, *A. nilotica* in East Africa from Sudan to Mozambique, and *A. pinnata* in most of Asia and the coast of tropical Africa (Sculthorpe, 1967; Lumpkin & Plucknett, 1980; Watanabe, 1982; Van Hove, 1989). In Tanzania, *A. pinnata* var. *africana* and *A. nilotica* were reported to be found in Lakes Victoria, Tanganyika, and Nyasa by Rendle (1907 in Mshigeni, 1982), Van Meel (1952 in Mshigeni, 1982), Demalsy (1953 in Lumpkin & Plucknett, 1980), and Demaret (1955 in Mshigeni, 1982). *Azolla nilotica* is also found in Dar es Salaam Region (Wagner, 1983, 1988, 1992).

V. Morphology

The *Azolla* macrophyte, called a frond, ranges from 1 cm to 2.5 cm in length in species such as *A. pinnata* and to 15 cm or more in the largest species, *A. nilotica*. It consists of a main rhizome, branching into secondary rhizomes, all of which bear small leaves alternately arranged. Unbranched, adventitious roots hang down into the water from nodes on the ventral surfaces of the rhizomes. The roots absorb nutrients directly from the water, though in very shallow water they may touch the soil, deriving nutrients from it. Each leaf consists of two lobes: an aerial dorsal lobe, which is chlorophyllous, and a partially submerged ventral lobe, which is colorless and cup-shaped and provides buoyancy. Each dorsal lobe contains a leaf cavity which houses the symbiotic *Anabaena azollae* (Peters, 1977; Lumpkin & Plucknett, 1980).

The interior surface of each leaf cavity is lined with an envelope (Peters, 1976) and covered by a mucilaginous layer of unknown composition which is embedded with filaments of *A. azollae* and permeated by multicellular transfer hairs (Shi & Hall, 1988). It has been shown that the mucilage is produced by the symbiont (Robins et al., 1986).

The blue-green alga *Anabaena azollae* consists of unbranched trichomes containing bead-like, heavily pigmented vegetative cells, approximately 6 µm in diameter and 10 µm in length (Van Hove, 1989), and lightly pigmented, intercalary heterocysts which are slightly larger and have thicker cell walls. In very young leaves, trichomes lack heterocysts. Heterocysts gradually increase in frequency until they comprise 30–40% of the algal cells (Van Hove, 1989). According to Hill (1977), heterocyst frequency reaches a maximum of about 30% of the cells in the 15th leaf from the apex. Mature trichomes also contain spores called akinetes. According to Peters (1975), trichomes, on average, consist of 60.9% vegetative cells, 23.1% heterocysts, and 16% akinetes.

No other algal species exists in the leaf cavities of the fern. However, small populations of non-nitrogen-fixing (Peters, 1977) bacteria do occur, such as *Pseudomonas* and *Azotobacter* (Bottomley, 1920; Shi & Hall, 1988) as well as *Arthrobacter* (Van Hove, 1989; Carrapico, 1991).

VI. Physiology

Physiologically, *Azolla–Anabaena* is outstanding because of its high productivity combined with its ability to fix nitrogen at substantial rates. It is capable of photosynthesizing at rates higher than most C4 plants since the variety of light-harvesting pigments contained in the two partners are complementary and can capture a wide range of wavelengths of light (Shi & Hall, 1988). Watanabe et al. (1977), conducting research at the International Rice Research Institute in the Philippines, have shown in laboratory studies that *Azolla* can double its mass in 3–5 days, growing in nitrogen-free solution, and can accumulate 30–40 kg N ha^{-1} in two weeks. In a rice paddy, five crops of *Azolla* were grown consecutively, producing 117 kg N ha^{-1} in 106 days. Under ideal conditions of light and temperature, Peters et al. (1980 in Watanabe, 1982) obtained doubling times of 2.0 days or less for *A. filiculoides*, *A. caroliniana*, *A. mexicana*, and *A. pinnata*.

The maximum biomass attained by *Azolla* spp., as reported by various researchers, is 3190 kg dry weight ha^{-1} for *A. caroliniana* and ranges from 640 to 2170 kg ha^{-1} for *A. pinnata*, from 830 to 1100 kg ha^{-1} for *A. mexicana*, and from 1700 to 5200 kg ha^{-1} for *A. filiculoides* (Watanabe, 1982).

There have been relatively few studies on *Azolla nilotica* in comparison with other *Azolla* species. However, Wagner (1996), conducting experiments in outdoor cement tanks, found that *A. nilotica* showed growth rates of from 25 to 90 g m^{-2} day^{-1} and attained a maximum biomass of 2.9 kg m^{-2}. This very high maximum biomass, in comparison with other species, is due to its large frond size and the tendency for its apices to extend vertically upwards. The shortest doubling time attained by *A. nilotica* was 2.0 days.

The reported rates of nitrogen fixation vary greatly. Becking (1979), examining the reports of many researchers working on various species of *Azolla* in various parts of the world, found that rates (in terms of ethylene production) ranged from about 20 to 200 μmol C_2H_4 g^{-1} dry weight $hour^{-1}$. Watanabe (1982) reported rates of 1.0–3.6 kg N ha^{-1} day^{-1}. In Indonesia, *Azolla* was found to fix 100–150 kg N ha^{-1} $year^{-1}$ (National Academy of Sciences, 1979). Wagner (1996) found rates of 116–695 nmol C_2H_4 g^{-1} fresh weight $hour^{-1}$ for *A. nilotica.*

Among types of organisms that fix nitrogen, symbiotic systems (e.g., legumes and *Azolla–Anabaena*) have the highest rates per unit area, followed by free-living blue-green algae, and then heterotrophic bacteria (Watanabe, 1985). Generally, *Azolla–Anabaena* is capable of fixing nitrogen at higher rates than legumes. Typical rates are approximately 400 kg N ha^{-1} $year^{-1}$ for the latter as compared to 1100 kg N ha^{-1} $year^{-1}$ for the former (Hall et al., 1995).

The nitrogen-fixing activity of the association is performed entirely by the symbiont, *Anabaena azollae.* Alga-free fronds are incapable of fixing nitrogen (Peters, 1977). In experiments performed on *A. azollae* isolated from the fern, it has been shown that about one-half of the nitrogen fixed by the alga is excreted as ammonium ion. In the intact association, the ammonium ions are assimilated by the enzyme glutamine synthetase, found within the fern, and converted to amino acids (Peters et al., 1977 in Watanabe, 1982). It appears that transfer hairs in the leaf cavities of *Azolla* play a role in the transport of ammonium ions to the cells of the host (Watanabe, 1982; Van Hove, 1989). The nitrogen incorporated into the *Azolla–Anabaena* complex is released into the medium upon mineralization (Watanabe et al., 1977).

The nitrogen-fixing ability of each leaf of *Azolla,* because it is a function of the heterocyst frequency of the symbiont it houses (Watanabe, 1982), varies with leaf maturity.

The relationship between *Azolla* and *Anabaena azollae* is one of permanent symbiosis—i.e., the two organisms are associated in all stages of the life cycle of the fern, and the association persists from one generation to the other regardless of whether reproduction is sexual or asexual (Van Hove, 1989). The packaging of *Anabaena* filaments into sporocarps is facilitated by branched epidermal trichomes known as sporangial pair hairs. The originally undifferentiated filaments become entrapped in a cup-shaped indusium which triggers their differentiation into akinetes. The symbiosis is reestablished following gametogenesis and embryogenesis (Peters & Perkins, 1993).

There is also evidence for the coevolution of host and symbiont. Cluster analysis showed that fatty acid composition of the symbionts were similar within the four *Azolla* spp. of subg. *Euazolla* and within the two *Azolla* spp. of the *Rhizosperma* (following the previous, widely accepted system of taxonomy). However, a separation occurred between symbionts from the two groups (Caudales et al., 1995).

It appears that certain of the bacteria present in the leaf cavities of the fern, particularly *Arthrobacter*, constitute a third partner in the *Azolla–Anabaena* symbiosis. The bacteria, apparently non-nitrogen-fixing, seem to be present throughout the life cycle of the fern, following a developmental pattern identical to that of *Anabaena azollae* (Van Hove, 1989; Carrapico, 1991). Forni et al. (1990) found that the strains of *Arthrobacter* that occurred in the megasporocarps were the same strains that occurred in the leaf cavities of the fern, indicating the permanence and consistency of the third partner in the relationship. The role of the bacteria in the partnership is not yet understood.

There have been a few reports that alga-free *Azolla* may rarely occur in nature (Fremy, 1930; Hill, 1977; Shi & Hall, 1988). Through various techniques, researchers have succeeded in obtaining alga-free *Azolla* (Nickell, 1958; Johnson et al., 1966; Moore, 1969; Lumpkin & Plucknett, 1980). However, since alga-free fronds cannot fix nitrogen, they develop extra roots and require nitrogenous fertilizer (Peters, 1976).

Anabaena azollae can be isolated from the fern (Peters & Mayne, 1974a; Peters, 1976). The isolate is still capable of fixing nitrogen, but at lower rates than when associated with the fern (Peters & Mayne, 1974b; Peters, 1975). Several scientists have succeeded in culturing the isolate (Ashton & Walmsley, 1976; Becking, 1976; Shi & Hall, 1988). However, the cultured isolate develops different characteristics from the fresh isolate, which has led to the proposal that it be called a "presumptive *A. azollae*" (Shi & Hall, 1988). Reintroduction of the cultured isolate into algae-free *Azolla* appears to be extremely difficult, though there is one report of success (Liu et al., 1984).

Although photosynthesis takes place in the vegetative cells of *Anabaena azollae*, there appears to be some dependency of the alga on its host. While in nonsymbiotic species of *Anabaena* the heterocysts never make up more than 3–5% of the cells, they compose up to 40% of the cells of the symbiont in mature *Azolla* leaves. From this it may be inferred that the carbon requirements of the symbiont are partially provided by the host. Again, it appears that transfer hairs play a role in transport of the nutrients (Van Hove, 1989).

VII. Reproduction

Azolla exhibits both sexual (involving a complex cycle) and asexual or vegetative reproduction (Lumpkin & Plucknett, 1980; Van Hove, 1989). When environmental conditions (not yet well-defined) are conducive, sexual reproduction occurs. At the first ventral leaf lobe initial of a lateral branch, instead of forming a leaf lobe, the fern produces two (four in *A. nilotica*) sporocarps which are of two types, the microsporocarps and the megasporocarps, which at each ventral site may be either of the same type or mixed. The microsporocarps, which are about 2 mm in diameter, each produce 8–130 microsporangia, each containing 32 or 64 spores aggregated into 3–10 massulae (Moore, 1969; Shi & Hall, 1988). The latter, because of their minute size, are known as microspores. The megasporocarps, which are about 0.5 mm in diameter, each produce a single megasporangium containing a single megaspore, which is much larger than a microspore. Each megaspore contains a small colony of *Anabaena azollae* filaments bearing akinetes.

At maturity, both the megasporocarps and the microsporocarps dehisce. The microsporangia break open, releasing spongy masses of microspores, or massulae, into the water. These massulae cling to the megaspores by filamentous structures known as glochidia (except in the case of *A. nilotica*) and usually sink to the bottom.

After a period of dormancy, each microspore germinates and grows into a prothallus that in turn produces ciliated, male gametes (antherozoids). The megaspore, still joined to the megasporocarp, germinates and develops into a prothallus that produces female gametes (oospheres). Oospheres are fertilized by antherozoids, thence developing into embryos. Each embryo germinates and pushes itself out of the sporocarp to form a plantlet, the sporophyte, that floats to the water surface. It takes one or two months from germination to produce a branched frond (Watanabe, 1982).

Despite significant advances in the study of the biology of *Azolla*, some important aspects of the life history remain unknown—e.g., the mechanism for the release of the antherozoids and the pathway of fertilization within the megaspore (Tan et al., 1986).

Asexual or vegetative reproduction, which is by far the most common means of reproduction both in nature and in agricultural application, consists of multiplication by simple fragmentation of the fronds. This occurs when the most developed secondary rhizomes, or branches, form abscission layers at their bases and break off from the main rhizome (Watanabe, 1982; Van Hove, 1989).

VIII. Environmental Factors Affecting *Azolla–Anabaena*

The environmental factors affecting *Azolla* have been reviewed by Lumpkin and Plucknett (1980, 1982), Hamdi (1982), Watanabe (1982), and Lumpkin (1987a, 1987b). Water is a fundamental requirement of *Azolla*. It can survive only a few days in a paddy field once the field is drained. Hechler and Dawson (1995) were the first to quantify the effects of drying. They found that nitrogenase activity in *A. caroliniana* was maximum when the moisture content of the tissue was 88–95% of the fresh mass; but under moisture stress, when moisture content dropped to 80%, nitrogenase activity decreased to less than one-fifth of the maximum. Therefore, proper water control in rice fields is essential for the utilization of *Azolla*. A shallow water depth of 5 cm or less is best, although *Azolla* can grow satisfactorily in greater depths.

The optimum temperature for *Azolla* spp. is between 18° and 28°C (Tuan & Thuyet, 1979), although some species can survive a very wide temperature range of about –5° to 35°C (Lumpkin, 1987a). According to Watanabe (1982), the optimum temperature for *A. pinnata*, *A. mexicana*, and *A. caroliniana* is about 30°C. Growth rates are reduced above 35°C and no species can survive at prolonged temperatures above 45°C (Lumpkin, 1987a). Hechler and Dawson (1995) found that, in *A. caroliniana*, nitrogenase activity could be detected at temperatures between 5° and 40°C, was relatively high at between 15° and 35°C, and was maximum at 25°C.

Azolla spp. generally grow best in less than full sunlight except in high latitudes during spring. Results of experiments differ according to the latitude where they were performed. In South Africa, Ashton (1974 in Lumpkin & Plucknett, 1980) found that growth and nitrogenase activity were highest at 50% of full sunlight. In the Niger basin, Kondo et al. (1989) found that the high light intensity and temperature prevailing during the hot season suppressed *Azolla* growth. On the other hand, low light intensities under a dense growth of rice cause *Azolla* to suffer or die (Lumpkin, 1987a). In exposing *A. pinnata* and *A. filiculoides* to 30–100% of full greenhouse sunlight in Australia, Cary and Weerts (1992) found the two species to have similar responses to shading. Biomass yields of cultures exposed to 30% sunlight were less than one-third of those exposed to full light. Hechler and Dawson (1995) found that nitrogenase activity in *A. caroliniana* was highest at photosynthetic photon flux densities of 400–1000 μmol m^{-2} $second^{-1}$ and declined rapidly at lower levels.

The optimum photoperiod for *Azolla* growth is 20 hours (Laurinavichene et al., 1990). Moreover, nitrogenase activity shows a definite diurnal pattern. Hechler and Dawson (1995) found that nitrogenase activity in *A. caroliniana* was very low early in the morning, rose sharply during midmorning, attained a maximum at 12:00–3:00 p.m., and then fell to a low level in the evening. Its diurnal pattern was determined to be due entirely to environmental factors, particularly temperature and light, and not due to an endogenous rhythm.

Besides affecting photosynthesis, light appears to regulate nitrogenase activity in *Azolla* independent of CO_2 fixation (Bar et al., 1991).

Optimum relative humidity for *Azolla* is about 85–90% (Watanabe, 1982). Higher humidity combined with high temperatures stimulate the growth of insects and fungi. Improvement in rice crop yield due to the application of *Azolla* is usually greater in a cool dry environment or season than during a wet environment or monsoon season (Lumpkin, 1987a). De Waha Baillonville et al. (1991) found that productivity of *Azolla* was much higher (twice as much with some ecotypes) in a subdesertic tropical area in Senegal than it was in the humid tropical islands of the Philippines, though the reason for this is as yet unknown. On the other hand, Hamdi (1982) reported that *Azolla* becomes dry and fragile at less than 60% relative humidity.

The optimum pH range for *Azolla* growth is 4.5–7, although it can survive within a range of 3.5–10 (Watanabe et al., 1977; Lumpkin & Plucknett, 1980; Lumpkin 1987b). However, the response of *Azolla* to pH is greatly affected by other factors of the environment such as light intensity, temperature, and soluble iron (Tuan & Thuyet, 1979; Lumpkin & Plucknett, 1980; Hamdi, 1982). At high light intensity (60,000 lux), optimum pH is 9–10, whereas at low light intensity (15,000 lux), optimum pH is 5–6. Nitrogen fixation was found to be optimal at a pH of 6.0 with a temperature of 20°C (Ashton, 1974, in Lumpkin & Plucknett, 1980). In greenhouse experiments, Cary and Weerts (1992) found that, at a water temperature of 25°C, both *A. pinnata* and *A. filiculoides* showed maximum growth at pH values of 5–7. *Azolla pinnata* showed greater tolerance to a wide pH range than did *A. filiculoides*, the latter growing much more poorly at pH values of 4 and 8.

Azolla requires all the essential elements that are required by other plants plus molybdenum (an essential constituent of nitrogenase) and cobalt, which are required for nitrogen fixation (Lumpkin, 1987b). Normally, nutrients must be available in the water, though in very shallow water *Azolla* may extract some nutrients from the soil. Moreover, adequate nutrient levels should be maintained throughout the period of growth. Tung and Shen (1985) found that, after an initial 4–6-week period of fast growth and high acetylene-reduction activity by *Azolla*, these two activities decreased markedly, more because of low nutrient levels in the water than because of shading by the rice.

Yatazawa et al. (1980) determined that the threshold levels of P, K, Mg, and Ca required in the medium for *Azolla* growth were approximately 0.03, 0.4, 0.4, and 0.5 mmol l^{-1}, respectively; whereas full nitrogenase activity required 0.03, 0.6, 0.5, and 0.5 mmol l^{-1}, respectively. The threshold levels of the micronutrients Fe, Mn, Mo, and B, for *Azolla* growth, were 50, 20, 0.3, and 30 $\mu g\ l^{-1}$, respectively.

Phosphorus is the most important and often limiting nutrient for *Azolla* growth (Lumpkin, 1987a, 1987b). Phosphorus deficiency is indicated by smaller, less vigorous plants and may cause the plants to become pink to deep red and fragile and to develop very long roots. Cells of *Anabaena azollae* become pale green and deformed.

When tested with phosphorus (H_2PO_4) nutrient levels of up to 40 mg P l^{-1}, Cary and Weerts (1992) found in greenhouse experiments that *Azolla pinnata* attained maximum biomass at 5 mg P l^{-1}, whereas *A. filiculoides* attained maximum biomass at 20 mg P l^{-1}. Kondo et al. (1989) found that growth and nitrogen content of *A. pinnata* (Niger isolate) was highest in laboratory culture at a phosphorus level of 3.1 ppm.

Although *Azolla* does not require any nitrogen in the medium, the level of nitrogen in the water does affect its growth and nitrogen-fixation rates. When tested with nitrogen (ammonium and nitrate) levels of up to 10 mg N l^{-1}, Cary and Weerts (1992) found that *A. pinnata* showed maximum growth at 1 mg N l^{-1}, whereas *A. filiculoides* showed maximum growth at 10 mg N l^{-1}. P. K. Singh et al. (1992) found that the relative growth rates of *A. caroliniana* and *A. pinnata* were higher at a nitrate level of 5 mM than they were in nitrogen-free medium. However, higher levels of nitrate reduced growth. Hechler and Dawson (1995) found that the source of nitrogen affected the response of *A. caroliniana*. Whereas 10.0 mg NO_3-N l^{-1} increased growth rate, the same concentration of ammonium-nitrogen decreased growth rate and urea had no effect.

Early reports indicated that the level of combined nitrogen in the medium had little or no effect on the nitrogen-fixation rate of *Azolla* (Peters, 1978; National Academy of Sciences, 1979; Lumpkin & Plucknett, 1980). It was thought that this was because the fern protects the endophyte from the effects of the medium. However, P. K. Singh et al. (1992) found combined nitrogen to adversely affect nitrogen-fixation rates. Nitrate levels of 5, 10, 15, and 20 mM caused significant observable inhibition of acetylene-reduction activity, as compared to the nitrogen-free controls, on the 16th, 12th, 8th, and 4th days after application, respectively. Hechler and Dawson (1995) found that 2.0 mg NH_4-N l^{-1} was sufficient to cause a significant reduction in nitrogenase activity in *A. caroliniana*, whereas 10.0 mg NO_3-N l^{-1} was required to reduce nitrogenase activity. On the other hand, 10.0 mg urea-N l^{-1} increased nitrogenase activity by 50%. Singh and Singh (1989) found that the application of nitrogen reduced heterocyst frequency in *Anabaena azollae*.

Iron is a common limiting element (Watanabe, 1982), since it is an essential constituent of nitrogenase (Fogg et al., 1973). Iron-deficient plants become yellow due to the depletion of chlorophyll. Roots become thin and whitish (Malavolta et al., 1981). The availability of iron is decreased by neutral to alkaline conditions (Lumpkin & Plucknett, 1980; Watanabe, 1982).

In calcium-deficient *Azolla*, there is intense reddening of the dorsal lobes and fronds become fragmented (Watanabe, 1982). Roots become short, thin, and light in color (Malavolta et al., 1981).

In potassium-deficient plants, fronds become yellowish brown (Watanabe, 1982). Roots become dark brown and their growth is stunted (Malavolta et al., 1981).

Cobalt is required for the symbiotic growth of *Anabaena azollae* in the host plant. In the absence of combined nitrogen, *Azolla* growth, chlorophyll content, and nitrogen fixation is increased by the addition of cobalt (Johnson et al., 1966).

Certain biotic factors also affect *Azolla*. It may be attacked by pests such as lepidopterous or dipterous insects or fungal diseases, particularly during hot, humid periods. These can be controlled, but such measures are costly (Hamdi, 1982; Lumpkin, 1987a). Snails are a common pest on *Azolla* (Kushari, 1991; Mochida, 1991). Pablico and Moody (1991) attempted to use the molluscicide Fentin acetate to control the golden apple snail, *Pomacea canaliculata* Lam. However, rates required to kill the snail (0.2–0.9 kg a.i. ha^{-1}) were phytotoxic both to *Azolla* and to rice. Rice seedling heights were reduced. When applied at 0.9 kg a.i. ha^{-1}, more than 90% of *A. pinnata* was killed.

Intraspecific competition also affects the performance of *Azolla*. Hechler and Dawson (1995) found that high plant density decreased specific nitrogenase activity per unit biomass and per unit area. They found that the optimal plant density for nitrogenase activity in *A. caroliniana* was 50–100 g dry weight m^{-2}.

When considering the utilization of *Azolla* during this age of modern industry, it is important to investigate its response to pollution of the environment. *Azolla* is extremely sensitive to SO_2 pollution in the atmosphere. Hur and Wellburn (1993) found that, even at concentrations as low as 25 nl l^{-1}, there was severe damage to fronds and significant reductions in growth, heterocyst development, and nitrogen-fixation rates. Ozone pollution also affects *Azolla*. Hur and Wellburn (1994a) found that growth rates of *A. pinnata* were decreased by 25%, 25%, and 50% when exposed for 1 week to 30, 50, and 80 nl O_3 l^{-1} of air, respectively. Exposure to ozone also reduced nitrogen-fixation rates and heterocyst frequency of the symbiont. Moreover, Hur and Wellburn (1994b) found that exposure to atmospheric NO_2 pollution decreased rates of growth, nitrogen fixation, heterocyst formation, and overall nitrogen cycling.

The use of different pesticides appears to have varying effects on *Azolla*. Ismail et al. (1995) found that molinate reduced the growth and nitrogenase activity of *A. pinnata* but increased its chlorophyll content. On the other hand, carbofuran significantly increased its chlorophyll content and nitrogenase activity but did not affect its growth.

IX. The Utilization of *Azolla–Anabaena*

Azolla–Anabaena has many uses. It can be utilized as a biofertilizer on rice and many other crops, an animal feed, a human food, a medicine, and a water purifier. It may also be used for the production of hydrogen fuel, the production of biogas, the control of weeds, the control of mosquitoes, and the reduction of ammonia volatilization that accompanies the application of chemical nitrogen fertilizer.

A. *AZOLLA–ANABAENA* AS A BIOFERTILIZER

Whereas research in past decades was aimed solely at maximizing agricultural yields, the present international focus is on combining increased productivity with environmentally sustainable agricultural practices (Peoples et al., 1995). In order for agriculture to be sustainable, nutrients must be replenished. Fortunately, for the all-important nutrient nitrogen this can be done through an environmentally favorable process, biological nitrogen fixation.

Nitrogen is the element most often limiting in food production (Hardy et al., 1968). Agricultural production is directly dependent on nitrogen. In rice, the amount of nitrogen absorbed to produce grain is nearly constant at 19–21 kg N t^{-1} of whole grain rice (Murayama, 1979). Therefore, yields can be raised significantly by increasing the amount of nitrogen available to crops. Nitrogen can be supplied to rice either by adding chemical fertilizers or by increasing the amount of nitrogen supplied naturally, particularly by biological nitrogen fixation.

The use of chemical fertilizers in rice fields is expensive, disturbs the equilibrium of agroecosystems, and causes pollution of the environment. These problems may be avoided by the use of biofertilizers (Madhusoodanan & Sevichan, 1992).

Most of the chemical nitrogenous fertilizer is produced by industrial nitrogen fixation. In this process, for every single unit of nitrogen fertilizer produced, two units of petroleum are required (Hamdi, 1982). This expensive mode of production, combined with the cost of transport, makes the application of fertilizer too expensive for the majority of farmers in developing countries. Moreover, petroleum is a limited, nonrenewable resource.

The use of nitrogenous fertilizers in agriculture has several effects on the environment. Nitrate-containing runoff from farmlands causes water pollution in surrounding aquatic ecosystems and contaminates groundwater. Nitrate concentrations above the safe level stipulated by the World Health Organization (11.3 mg l^{-1}) have been reported in drinking water in England, France, Denmark, Germany, the Netherlands, and the United States. Mixing infant formula with nitrate-contaminated water can be fatal to newborn babies (Cunningham & Saigo, 1990).

The use of nitrogenous fertilizers causes acidification of soils (Stumpe & Vlek, 1991) and their long-term application significantly reduces microbial activity in the soil (Sutton et al., 1991).

Not only the use of, but also the production of, fertilizers causes pollution. Pasternak et al. (1988) reported that growth characteristics of trees are affected by atmospheric pollution from nitrogen fertilizer factories.

The utilization of biofertilizers has several advantages over chemical fertilizers. First, biofertilizers are inexpensive, making use of freely available solar energy, atmospheric nitrogen, and water. Second, biofertilizers utilize renewable resources, whereas the production of chemical fertilizers depends on petroleum, a diminishing resource. Third, biofertilizers are nonpolluting. Fourth, besides supplying nitrogen to crops, biofertilizers also supply other nutrients such as vitamins and growth substances (Venkataraman & Kaushik, 1980). Fifth, biofertilizers improve the general fertility of the soil by increasing the availability to crops of a number of nutrients, by increasing the organic matter in soil, and by improving soil structure.

Although there are no published reports concerning the release of growth-promoting substances by *Azolla–Anabaena,* this aspect deserves attention. There have been several studies on the effect of hormones, vitamins, and other growth-promoting substances, extracted from free-living blue-green algae, on the growth of various crops including rice (Misra & Kaushik, 1989a, 1989b; Wang et al., 1991). Therefore, there exists the possibility that *Anabaena azollae* may also produce such substances.

Azolla–Anabaena can be beneficial as a biofertilizer on a variety of crops, including rice, taro, wheat, and many others.

There are three primary methods for applying *Azolla* to crops. First, it may be grown in the field as a monocrop during the fallow season and then incorporated into the soil before planting the target crop. Second, *Azolla* may be grown as an intercrop among the target crop. Third, natural or deliberately cultured growths of *Azolla* may be harvested from ponds, swamps, or flooded fields and applied to a variety of target crops, either by incorporating it into the soil before planting the crop or by applying it as a mulch on top of the soil around the bases of crop plants. Often, a combination of these methods of application is utilized.

B. THE UTILIZATION OF *AZOLLA–ANABAENA* ON RICE

The most suitable target crop for the application of *Azolla* is lowland rice, since both plants require similar environmental conditions—most notably, a flooded habitat—and they grow together compatibly.

Azolla may be applied on rice either as a monocrop or an intercrop. As an intercrop it is usually inoculated into the field just after transplanting the rice and, after a period of growth, may be incorporated into the mud or allowed to die naturally by fungal rot or light starvation (Lumpkin, 1987a). However, a combination of applications is usually recommended. In particular, benefit to rice can be maximized by first growing *Azolla* as a monocrop, incorporating it, and then, after transplanting the rice, applying it again as an intercrop with one or more subsequent incorporations. For many centuries, *Azolla* has been used to successfully increase rice yield in Vietnam and southern China (Fogg et al., 1973; Watanabe & Liu, 1992). The earliest written record of *Azolla* was in an ancient Chinese dictionary called the *Er Ya*, which appeared about 2000 years ago. A Chinese book on agricultural techniques published by Jia Si Xue in 540 a.d. mentioned *Azolla* with respect to applied plant cultivation (Shi & Hall, 1988). *Azolla* grows in lowland rice fields in Indonesia, India, China, Vietnam, Thailand, Senegal, and other tropical countries.

Many experiments have demonstrated the effectiveness of *Azolla* as a biofertilizer on rice, though the extent of the benefit varies greatly according to the climate, the method of application, the species of *Azolla* used, and many other factors. At Davis, California, the use of *Azolla* increased rice yields by 112% over unfertilized controls when applied as a monocrop during the fallow season, by 23% when applied as an intercrop with rice, and by 216% when applied both as a monocrop and an intercrop (Peters, 1978). Tung and Shen (1985) found that *Azolla* grown with rice appeared to suppress the growth of rice in the early stages, probably due to competition. However, at maturity, although rice grown with *Azolla* did not have greater height or tiller number, straw and grain yield were higher, particularly grain yield which was 42–55% higher than the controls where no *Azolla* was applied. Sisworo et al. (1990) found that *Azolla* was equally as effective as urea on rice when both were applied at the rate of 30 kg N ha^{-1} at transplanting and at maximum tillering. In the Niger basin, Kondo et al. (1989) applied *A. pinnata* as an intercrop, inoculating it 5 days after transplanting the rice and then incorporating it and reinoculating 27 days after transplanting. They obtained a 27% increase in grain yield. In China, the application of *Azolla* as a monocrop and as an intercrop incorporated 40 days after transplanting resulted in a 30.6% increase in rice grain yield (Lay et al., 1989). In Tanzania, Wagner (1996) applied *A. nilotica* in various trials as an intercrop, with one incorporation, obtaining increases of 19–103% in grain yield and of 1–23% in straw yield. Significant increases were also found in rice height and the number of tillers per hill.

In contrast with the application of chemical nitrogenous fertilizers, the benefits brought about by green manures such as *Azolla* and *Sesbania* sp. are long-term. In an experiment where 10 crops of rice were planted consecutively, application of these green manures, both as a monocrop and as an intercrop, increased grain yield by 1.8–3.9 t ha^{-1} above the controls, an effect similar to or greater than the effect of 60 kg N ha^{-1} urea application. The green manures were found to have beneficial residual effects after nine crops of rice, whereas urea fertilizer did not (Ventura & Watanabe, 1993).

Singh and Singh (1990a) found that *Azolla* application improves soil fertility by increasing total nitrogen, organic carbon, and available phosphorus in the soil. These findings are supported by Satapathy (1993) and Thangaraju and Kannaiyan (1993), who found that the most effective application for increasing soil fertility was first culturing *Azolla* as a monocrop, incorporating it before transplanting, and subsequently culturing it as an intercrop with two incorporations. Van Hove (1989) found that *Azolla* improves soil structure when incorporated because of its high productivity, which supplies large quantities of organic matter.

Wagner (1996) conducted experiments in which *Azolla nilotica* was applied to rice grown in outdoor cement tanks with soil collected from paddy, closely simulating natural rice paddy ecosystems. Kjeldahl analysis was used in measuring the nitrogen content of all components in the tanks at the commencement and termination of the experiments. The results showed that *Azolla* had a significant positive effect on total nitrogen over time.

Another benefit of applying *Azolla* as a biofertilizer is that in low-potassium environments it has a greater ability to accumulate potassium than does rice. Thus, when the fern decomposes, it acts indirectly as a potassium fertilizer (Van Hove, 1989).

Many studies have been conducted that compare the effectiveness of *Azolla* with other types of biofertilizers. In a study on the effects of free-living blue-green algae (*Tolypothrix tenuis*, *Aulosira fertilissima*, *Nostoc* sp., *Anabaena* sp., and *Plectonema* sp.), *Azolla*, and chemical nitrogenous fertilizer, S. Singh et al. (1992) found that rice grain yield was highest with the application of *Azolla* + 120 kg N ha^{-1} (5.01 t ha^{-1}), followed by blue-green algae + *Azolla* + 60 kg N ha^{-1} (4.62 t ha^{-1}) and, lastly, by 120 kg N ha^{-1} (4.61 t ha^{-1}).

In a long-term experiment at the International Rice Research Institute in the Philippines (Watanabe & Ventura, 1992), results showed that *Azolla* gave higher rice grain yield (3.3–3.9 t ha^{-1}) during the dry season than did *Sesbania* or urea (both 1.8–2.5 t ha^{-1}). During the wet season there was no difference among *Azolla*, *Sesbania*, and urea treatments, though each of these yielded 1.7 t ha^{-1} more than the control. Residual effects were shown with both biofertilizers but not with urea. Both biofertilizers increased soil organic matter content.

Singh and Singh (1990b) found that the addition of phosphorus fertilizer increased growth and nitrogen fixation of both blue-green algae and *Azolla* biofertilizers. Whereas the application of either *Azolla* or blue-green algae, along with the addition of phosphorus, increased grain and straw yield of rice, the addition of phosphorus alone had no effect. The effect of *Azolla* on rice was similar to that of 30 kg N ha^{-1}, while the effect of blue-green algae was comparatively less.

Lumpkin (1987a) discussed the maintenance of *Azolla* in nurseries and its cultivation and field management. Since sexual reproduction is rare in some ecotypes (and the environmental conditions that trigger it are not well known) and since the production of a large biomass of *Azolla* from spores is a slow and difficult process (Van Hove, 1989), spores are not normally used as planting material. Vegetative material generally must be used. Therefore, cultures of *Azolla* must be maintained in nurseries throughout the year. During the off-season, it can be cultured in ponds, canals, or paddy fields, but care must be taken to protect it from death or injury by cold in temperate climates during overwintering or by heat in the tropics and in temperate climates during oversummering. The water should be changed, or cold water added, if the water temperature rises above 35°C. For the purpose of shading, tall herbaceous plants can be planted around the edge of the nursery or the *Azolla* can be intercropped with rice, *Pistia stratiotes*, *Eichhornia crassipes*, or *Alternanthera philoxeroides* (Lumpkin, 1987a).

To overcome the problem of maintenance of *Azolla* cultures during winter or summer, a technique of harvesting and seeding *Azolla* sporocarps was developed in China (Lu, 1987). However, this was successful with only a few strains, and growth in seedbeds was slow, so this technique has not become widely used. More recently, a simple technique was developed to produce dried sporocarp inocula which can remain viable for two years (Kannaiyan, 1993). Nevertheless, vegetative propagation is still the common practice. The utilization of tolerant strains, particularly strains of *A. caroliniana*, can be a solution to this problem in areas where there are dry or cool periods (Watanabe & Liu, 1992).

Just prior to the rice-growing season, *Azolla* must be multiplied in large quantities in preparation for field cultivation (Hamdi, 1982; Lumpkin, 1987a). This can be done in extensions of the nurseries, canals, ponds, or fields. During multiplication, *Azolla* mats must be continually subdivided in order to prevent competition for light, space, and nutrients and thus maintain a rapid growth rate.

The level of inoculation of *Azolla* into fields varies from 25 to 800 g m^{-2}, depending on the length of time it may be grown as a monocrop or intercrop, the availability of inoculum, and the labor costs of frequent incorporation. A high level of inoculation (500–800 g m^{-2}) followed by subdivision and partial incorporation every 2–4 days, keeping a linear growth phase, gives the best results (Lumpkin, 1987a).

When applying *Azolla* as an intercrop, early inoculation (5 days after transplanting) is preferable, since it controls weeds and gives significantly higher yields of rice grain and straw than does late inoculation (30 days after transplanting) (Satapathy, 1993).

As mentioned above in section VIII, phosphorus is often the limiting nutrient to growth and nitrogen-fixing activity of *Azolla*. Therefore, in its utilization as a biofertilizer, the application of phosphorus is usually recommended. Although Watanabe and Ramirez (1984) found that the growth of *A. pinnata* was satisfactory without adding phosphate fertilizer in soils having Olsen P (available phosphorus) values greater than 30 mg kg^{-1}, and phosphorus sorption capacity lower than 1500 mg P_2O_4 (100 $g)^{-1}$, for most paddy soils, the addition of phosphorus gives beneficial returns.

The recommended amount and method of application of phosphorus varies greatly. Watanabe et al. (1980) recommended 6 split applications of 2.5 kg P_2O_4 ha^{-1}, making a total of 15 kg P_2O_4 ha^{-1}. Lumpkin (1987a) recommended an application level of 0.5–1.0 kg P ha^{-1} day^{-1}, applied in a very soluble form such as phosphoric acid or triple superphosphate, particularly by gradually dripping the phosphorus solution into the water. He also recommended a water depth of 2–3 cm, which allows the *Azolla* roots to absorb phosphorus by contact with the soil. In Niger, Kondo et al. (1989) found that a field application of 6.5 kg P ha^{-1} per 13 days gave maximum growth of *A. pinnata*. For every 1 kg of superphosphate added, 3.66 kg N were produced by *Azolla*. Singh and Singh (1988) found that heterocyst frequency of *Anabaena azollae* and nitrogen fixation and biomass accumulation of *Azolla* were all higher with split applications of phosphorus than with a single application and were the least with no phosphorus application.

Watanabe et al. (1988) showed that phosphorus-enriched *Azolla*, inoculated into rice fields, was capable of multiplying 5–7 times before it became phosphorus deficient. They found the best method of enrichment was to apply phosphorus at the rate of 4.33 kg P/ha (10 kg P_2O_5/ha) twice at 2-day intervals and then, after 3 days, to harvest the *Azolla* for application on rice. Subsequent application of phosphorus in the field once or twice 2 weeks after inoculation further accelerated biomass production of the *Azolla*.

Singh and Singh (1995) found that both the phosphorus enrichment of *Azolla* inoculum and the application of phosphorus fertilizer during intercropping increased nitrogen uptake by rice, grain yield, and straw yield. The differences between the benefits of enriched and unenriched *Azolla* decreased, however, with increasing levels of phosphorus applied during intercropping.

The high phosphorus requirement of *Azolla* sometimes restricts its application in areas where soils have low nitrogen content and farmers are unable to purchase phosphate fertilizer. One possible solution could be the development of mutant strains of *Azolla* that have a low phosphorus requirement. Vaishampayan et al. (1992) developed a mutant strain with a phosphorus requirement of only 0.75 mM, while common strains of *Azolla* species generally required 1.5–2.0 mM phosphate. Different species of *Azolla* also differ in their phosphorus requirements. Kushari and Watanabe (1992) showed that *A. pinnata* was less affected by phosphorus-deficient conditions than was *A. microphylla*, *A. mexicana*, *A. filiculoides*, and *A. nilotica*.

It is important to note that when *Azolla* is incorporated and decomposes, the same phosphorus that was applied as fertilizer is then made available for uptake by the rice crop (Lumpkin, 1987a).

In certain deficient soils, the addition of potassium, lime, molybdenum, or iron may also enhance the growth and nitrogen-fixing activity of the *Azolla* biofertilizer (Rains & Talley, 1979; Lumpkin, 1987a).

Nitrogenous fertilizers are also sometimes applied along with *Azolla*. These can be used to supplement the contribution of nitrogen by *Azolla* with little or no interference with its growth and nitrogen-fixation processes. However, in soils that are rich in nitrogen and phosphorus, the use of *Azolla* alone is best, since the addition of nitrogen fertilizer as well tends to reduce rice grain yield, though it may increase straw yield (Joy & Havanagi, 1990).

It has been shown that an *Azolla* cover in a rice field reduces by 20–50% the ammonia volatilization that occurs following the application of inorganic nitrogen fertilizers. This is due to the fact that the *Azolla* cover reduces light penetration into the floodwater, thus hindering the rise of pH which normally stimulates ammonia volatilization in an *Azolla*-free rice field (Watanabe & Liu, 1992).

Most of the nitrogen fixed becomes available to rice only after the *Azolla* has decomposed, although a small amount of ammonium is released into the water by *Azolla* during growth (Moore, 1969; Silvester, 1977; National Academy of Sciences, 1979; Watanabe, 1984). Chung-Chu (1984) determined that 3–4% of the total nitrogen fixed by *Azolla* is excreted into the medium during its growth.

During decomposition, organic nitrogen is mineralized rapidly during the first two weeks and then at a more gradual rate (Watanabe, 1984). Nitrogen is released mainly in the form of ammonium. Ammonium-nitrogen released was found to stabilize at about 1 mg ammonium-N g^{-1} of fresh *Azolla*, which was 26–28% of the total nitrogen content of *Azolla* (Tung & Shen, 1985).

Rosenani and Chulan (1992), working with *Azolla pinnata*, found that when *Azolla*-nitrogen (labeled with ^{15}N isotope) was applied to rice at transplanting, the recovery of the nitrogen by the crop was only 20.2%; when it was applied at maximum tillering (30 days after transplanting), the nitrogen recovery was 30.2%. Recovery of urea-nitrogen was similar, being 22.5% and 38.6% for application at the same respective stages. However, in the succeeding rice crop, recoveries were more than twice as much from *Azolla* (4.0–4.9%) as from urea (1.9–2.1%).

Incorporation of *Azolla* into the soil improves the release of nitrogen (Tung & Shen, 1985). If *Azolla* is grown as a monocrop, the field should be drained several days in advance of incorporation. The last mat should be incorporated and the field kept drained for 4 or 5 days before transplanting rice in order to speed decomposition (Lumpkin, 1987a). As an intercrop, *Azolla* incorporated 78 days after transplanting rice was shown to contribute a greater amount of nitrogen to rice grain than was contributed by earlier incorporation (30–53 days after transplanting) (Ito & Watanabe, 1985). Since it has been found that the optimal stocking density for *Azolla*, with respect to area-specific nitrogenase activity, is approximately 50–100 g dry weight m^{-2} (Hechler & Dawson, 1995), nitrogen inputs may be best maximized by frequent but partial incorporations of *Azolla*.

When comparing the incorporation of three species of *Azolla*, Tung and Shen (1985) found that *A. pinnata* underwent more complete mineralization than *A. filiculoides*, followed by *A. mexicana*. *Azolla filiculoides* and *A. nilotica* have more lignin in their mature fronds than do other species, and they therefore have slower decomposition rates.

A first crop of rice was found to recover only 15–20% of the nitrogen in *A. filiculoides* that had been labeled with ^{15}N and incorporated into the soil (Lumpkin, 1987a). Ventura et al. (1992) found that *Azolla* fronds with high nitrogen content showed faster and greater nitrogen mineralization than did fronds having low nitrogen content, regardless of the species. Of four species tested under the same conditions, *A. microphylla* showed the greatest nitrogen content, while *A. pinnata* var. *pinnata* showed the lowest. The effectiveness of *Azolla* as a biofertilizer is determined primarily by its nitrogen content, which, in turn, depends on the level of phosphorus nutrition and the species of *Azolla.*

Incorporation of *Azolla* into the soil also enhances the release of other nutrients. Mian and Azmal (1989) found that when *Azolla* was cultured with rice and incorporated, about 28% of the phosphorus present in *Azolla* was subsequently taken up by rice plant tissue.

Recently, research has been conducted on sexual hybridization in an effort to improve the performance of *Azolla* (Watanabe & Liu, 1992). Hybridization between *A. microphylla* and *A. filiculoides* (male) improved annual biomass production. In the parent material, the latter grew better in the spring, while the former grew better in the summer and autumn due to its higher-temperature tolerance. The hybrid, however, produced biomass comparable to that of *A. filiculoides* in the spring and comparable to that of *A. microphylla* in the summer and autumn, thus boosting overall annual production. Van Cat et al. (1989) showed that hybrids between these same two species had stem lengths that were intermediate between those of the parents. The hybrid did not show stress (red color) under phosphorus- or calcium-deficient conditions and had higher nitrogen content than did the parent *A. microphylla.* Biomass production in the field was higher than that of *A. microphylla.*

There has also been some success in transferring *Anabaena* from one species of *Azolla* to another (Watanabe & Liu, 1992). Transferring *Anabaena* from temperature-tolerant *A. microphylla* to *Anabaena*-free *A. filiculoides*, resulted in high-temperature tolerance in the latter, indicating that heat tolerance may be partly controlled by the symbiont.

Such recent achievements in sexual hybridization indicate that genetic enhancement may be the key to improving some of the characteristics that limit *Azolla* utilization, such as sensitivity to high temperature, a high phosphorus requirement, and susceptibility to insect attack (Watanabe & Liu, 1992).

In summary, the characteristics that make *Azolla* suitable as a biofertilizer on rice are as follows:

1. A shallow, freshwater habitat such as is found in a flooded rice field is the ideal environment for *Azolla.*
2. *Azolla* fixes nitrogen at substantial rates.
3. *Azolla* has rapid growth.
4. Since *Azolla* floats at the water surface, it cannot compete with rice for light and space.
5. In most climates, *Azolla* grows best under a partial shade of vegetation which a rice canopy, in its early and intermediate stages of growth, can easily provide.
6. When rice approaches maturity, due to low light intensities under the canopy and depletion of nutrients, *Azolla* begins to die and decompose, thus releasing nutrients into the medium.
7. Azolla decomposes rapidly, and therefore the nitrogen it has fixed and the phosphorus and other nutrients it may have absorbed from the water, perhaps in competition with the rice, are rapidly released back into the medium and made available for uptake by rice during grain development.

8. *Azolla* has a greater ability than rice to accumulate potassium in its tissues in low-potassium environments; thus, after decomposition, it makes this nutrient available to rice.
9. In contrast with chemical nitrogenous fertilizers, *Azolla* has long-term residual effects, in particular, the improvement of soil fertility by increasing total nitrogen, organic carbon, available phosphorus, potassium, and other nutrients.
10. If chemical nitrogenous fertilizers are applied, an *Azolla* mat reduces the volatilization of ammonia that normally occurs.
11. A thick *Azolla* mat in a rice field has the side-benefit of suppressing weeds.

C. THE APPLICATION OF *AZOLLA-ANABAENA* ON CROPS OTHER THAN RICE

Azolla can be beneficial to many target crops other than rice. Particularly, any agricultural crop that grows in waterlogged conditions can be a suitable target crop for *Azolla*. Noteworthy among these are taro and its relatives. In China, *Azolla* is reported to be used as a green manure for taro (*Colocasia esculenta*) (Anonymous, 1982). Teckle-Haimanot (1995), experimenting with the utilization of *Azolla mexicana* on taro in the Cook Islands, found that the incorporation of *Azolla* into the mud and subsequent intercropping with *Azolla* resulted in 54.6% greater yields than the control, while taro intercropped with *Azolla* in slowly flowing water gave yields that were 87.3% greater than the control. Both *Azolla* treatments gave significantly higher yields than did treatments fertilized with chemical nitrogen and phosphorus.

Azolla is also beneficial to wheat when applied in a rotating rice–wheat cropping system (Kolhe & Mittra, 1990). *Azolla* applied as a monocrop between the wheat and rice crops, or applied as an intercrop with rice, has a significant beneficial effect on subsequent wheat crops. Mahapatra and Sharma (1989) found that the application of *Azolla* with *Sesbania* had beneficial residual effects on subsequent wheat crops, raising grain yield by 56–69% over controls.

The third method of application of *Azolla* mentioned in section IX.A—namely, the harvest of natural or deliberately cultured growths of *Azolla* from any adjacent water body for application on the target crop—can be utilized for virtually any agricultural or ornamental crop. Sculthorpe (1967) reports this practice from tropical Africa, India, and Southeast Asia. Marwaha et al. (1992) found that this method of application of *Azolla* (especially fresh fronds) increased grain yield of wheat, though straw yield and the number of tillers per plant were largely unaffected.

The method is also practiced in Senegal (Van Hove, 1989), where a succession of vegetable crops are planted on the banks of ponds. Between crops, *Azolla* is harvested from the ponds and incorporated into the soil. In the case of permanent crops such as bananas, *Azolla* is applied as a mulch on the soil surface around the bases of the plants. When there is an overproduction of *Azolla*, it can be mixed with soil and rice straw to form compost. Superphosphate may be added to reduce nitrogen loss (Van Hove, 1989).

Ram et al. (1994) found that the incorporation of 6, 12, 18, and 24 t ha^{-1} of fresh *Azolla* into the soil significantly increased its water-holding capacity, organic carbon, ammonium-nitrogen, nitrate-nitrogen, and its available phosphorus, potassium, calcium, and magnesium, while it decreased pH and bulk density. Such incorporation significantly raised the yield of mungbeans.

D. OTHER USES OF *AZOLLA-ANABAENA*

Azolla is used as a food supplement (fresh or dried or as silage) for a variety of animals, including pigs (in China), rabbits, chickens, and ducks (in Senegal and Ivory Coast), and fish (in China) (Van Hove, 1989). Sculthorpe (1967) reports that *Azolla* is harvested in large quantities from water bodies in parts of tropical Africa, India, and Southeast Asia and utilized as fodder for cattle and pigs. Ali and Leeson (1995), experimenting with broilers, found that the use of *Azolla* feed resulted in growth and body weight values similar to those resulting from the use of maize-soyabean meal.

In a study on lactating cows, Nik-Khah and Motaghi-Talab (1992) found that *Azolla* could be used as a feed ingredient (constituting up to 35%) with milk yields and fat percentages being maintained at the same levels as with conventional feeds. Production levels, however, were not increased.

Das et al. (1994) found that digested *Azolla pinnata* slurry remaining after biogas production (see below) was suitable as a fish pond fertilizer, significantly increasing phytoplankton populations in comparison with either digested or raw cow dung. Moreover, they found that conventional fish feed and digested *Azolla* slurry, mixed in the ratio of 4:1, resulted in the highest growth rate of fish. El-Sayed (1992), on the other hand, found that supplementing the diet of both fingerling and adult Nile tilapia, *Oreochromis niloticus* L., with *Azolla pinnata* had a negative effect on growth of the fish.

A rice–*Azolla*–fish culture system has proven to be quite successful in Fujian, China (Watanabe & Liu, 1992). Pits and ditches are dug in the rice field with double narrow row planting of rice. This allows space for fishing without reducing rice yield. Several strains of *Azolla* are inoculated and three or more kinds of fish (herbivorous and omnivorous) are mixed in proper ratios. A supplement of fish feed is sometimes necessary. This system has been shown to result in 27% recovery of *Azolla* nitrogen by fish and 23% recovery by rice, with 35% incorporation into soil and floodwater (15% loss). By comparison, a simple rice–*Azolla* system resulted in 26% recovery of *Azolla* nitrogen by rice, with 35% incorporation into soil and floodwater (39% loss). Potassium was also recycled effectively by the rice–*Azolla*–fish system. Moreover, the occurrence of diseases and insect pests was reduced. Thus, this system reduces expenses by reducing the inputs of fertilizer and pesticides required, increases farmers' incomes, and increases ecological stability.

Azolla appears to be fit for human consumption. A few researchers have experimented with the preparation of *Azolla* in soup or "*Azolla*-meat" balls as food for man. However, such recipes are as yet unpublished (Van Hove, 1989). Li Shi-zhen published a book in China in the 16th century that described the medicinal properties of *Azolla* (Shi & Hall, 1988). In Tanzania, *Azolla* has been reported to be used effectively as a traditional cough medicine (Wagner, 1996).

Another potential yet undeveloped use of *Azolla* is for the production of hydrogen, a nonpolluting, high-energy fuel. When *Azolla–Anabaena* is grown in a nitrogen-free atmosphere and/or a water medium containing nitrate, the nitrogenase in the symbiont, instead of fixing nitrogen, evolves hydrogen, using water as the source (Peters, 1975, 1976; Newton, 1976). Newton (1976) recorded hydrogen production at a rate of 760 nmol H_2 g^{-1} fresh weight $hour^{-1}$.

More-recent studies (Hall et al., 1995) have shown that rates of hydrogen production can be increased by exposure to a microaerobic environment, a partial vacuum, or argon-enriched or carbon dioxide-enriched atmospheres or by immobilization of cells of *Anabaena azollae* isolated from the fern. Cells may be immobilized by entrapment in transparent or translucent gels or polymers in order to increase the functional life time of the cells. Using a "trickling-medium" column bioreactor, Park et al. (1991) obtained a production rate of 83 ml H_2 g^{-1} Chl day^{-1}.

Some researchers, particularly in the Philippines, have investigated the use of *Azolla* in the production of biogas. Anaerobic fermentation of *Azolla* (or a mixture of *Azolla* and rice straw) results in the production of methane gas which can be utilized as fuel. Moreover, the remaining effluent can be used as a fertilizer because it contains all the nutrients originally incorporated in the plant tissues except for a small percentage of nitrogen lost as ammonia (Van Hove, 1989). Das et al. (1994) mixed cowdung and *Azolla pinnata* residues and found that the best ratio was 1:0.4, which gave a gas production 1.4 times that of cowdung alone.

Azolla can be used for the control of weeds. Krock et al. (1991) found that an *Azolla* cover significantly reduced the total amount of weeds, particularly the predominant weed *Monochoria vaginalis*, though grasses and hedges could not always be controlled. The *Azolla* cover reduced light intensity by about 90%, reducing photosynthesis in the floodwater and thus reducing oxygen concentration of the water by more than 50%. Besides reducing light intensity, an *Azolla* cover alters light quality, the green leaves having a filter effect that increases the relative amount of infrared rays. This can hinder the germination of light-sensitive seeds.

Azolla can also be used in the control of mosquitoes, for a thick *Azolla* mat on the water surface can prevent breeding and adult emergence. In a survey of pools, ponds, wells, rice fields, and drains, Ansari and Sharma (1991) found that breeding by *Anopheles* spp. was almost completely suppressed in water bodies that were completely covered with *Azolla*. Breeding of *Culex* spp. was not completely inhibited but was reduced. Rajendran and Reuben (1991) found that immature mosquito populations of *Anopheles subpictus* Grassi, *Culex pseudovishnui* Colless, and *C. tritaeniorhynchus* Giles were reduced by a 90% cover of *Azolla microphylla*. However, they concluded that although *Azolla* might be useful in controlling mosquitoes in long-term rice, it might be of little value in short-term and medium-term rice: peak densities of larvae occur during the second week after transplanting rice, but only 80% coverage by *Azolla* can be achieved 13–14 days after transplanting. Laboratory tests (Rajendran & Reuben, 1988) showed that *A. pinnata* greatly reduced both oviposition and adult emergence of *Culex quinquefasciatus* Say and *Anopheles culicifacies* Giles, but not larval survival. Egg hatchability was partially reduced in the latter.

There is also a possibility that *Azolla* could be used for purifying polluted water. Jain et al. (1989) found that *A. pinnata* and *Lemna minor* (duckweed) removed the heavy metals iron and copper from polluted water if present at low concentrations. They suggested that effluent containing such pollutants at low concentrations could be treated by passing it through ponds containing one or both of these water plants. Saxena (1995) found that a mixed culture of *Lemna* and *Azolla* in the ratio of 2:1 was able to sufficiently purify highly polluted effluent from a factory to the extent that it could be used for agricultural purposes.

X. Concluding Remarks

In this age when mankind is threatened by drastic global environmental changes triggered by his own activities, we need to investigate and develop alternative strategies for conducting our affairs. The use of sustainable and environmentally appropriate practices in agriculture can greatly contribute to the ecological stability of the planet. The application of *Azolla* as a biofertilizer on agricultural crops, reducing or replacing chemical fertilizers, can play a significant role in maintaining or improving the state of the global environment. Several other of its uses may also be very beneficial to man. Further collaborative efforts in research are required to make the best use of this important natural resource, this "green gold mine."

XI. Acknowledgment

I would like to express my deepest gratitude to Prof. Keto E. Mshigeni for his experienced guidance and support and for his example of academic excellence.

XII. Literature Cited

Ali, M. A. & S. Leeson. 1995. The nutritive value of some indigenous Asian poultry feed ingredients. Anim. Feed Sci. Technol. **55:** 227–237.

Anonymous. 1982. China and the West: Sharing solutions to agricultural problems. World Farm. **24:** 76–78.

Ansari, M. A. & V. P. Sharma. 1991. Role of *Azolla* in controlling mosquito breeding in Ghaziabad District villages (U.P.). Indian J. Malariol. **28:** 51–54.

Ashton, P. J. & R. D. Walmsley. 1976. The aquatic fern *Azolla* and its *Anabaena* symbiont. Endeavour **19:** 39–43.

Bar, E., S. A. Kulasooriya & E. Tel-or. 1991. Regulation of nitrogenase activity by light in the *Azolla–Anabaena* symbiosis. Bioresource Technol. **38:** 171–178.

Becking, J. H. 1976. Contributions of plant–algal associations. Pages 556–580 *in* W. E. Newton & C. J. Nyman (eds.), Proceedings of the 1st International Symposium on Nitrogen Fixation. Vol. 2. Washington State University Press, Pullman.

———. 1979. Environmental requirements of *Azolla* for use in tropical rice production. Pages 345–373 *in* International Rice Research Institute, Nitrogen and Rice. International Rice Research Institute, Los Banos, Philippines.

Bottomley, W. B. 1920. The effect of organic matter on the growth of various water plants in culture solution. Ann. Bot. **34:** 353–365.

Carrapico, F. 1991. Are bacteria the third partner of the *Azolla–Anabaena* symbiosis? Pl. & Soil **137:** 157–160.

Cary, P. R. & P. G. J. Weerts. 1992. Growth and nutrient composition of *Azolla pinnata* R. Brown and *Azolla filiculoides* Lamarck as affected by water temperature, nitrogen and phosphorus supply, light intensity and pH. Aquatic Bot. **43:** 163–180.

Caudales, R., J. M. Wells, A. D. Antoine & J. E. Butterfield. 1995. Fatty acid composition of symbiotic cyanobacteria from different host plant (*Azolla*) species: Evidence for coevolution of host and symbiont. Intl. J. Syst. Bacteriol. **45:** 364–370.

Chung-Chu, L. 1984. Recent advances on *Azolla* research. Pages 45–54 *in* W. S. Silver & E. C. Schroder (eds.), Practical application of *Azolla* for rice production. Martinus Nijhoff/W. Junk, Dordrecht.

Cunningham, W. P. & B. W. Saigo. 1990. Environmental science: A global concern. Wm. C. Brown, Dubuque, Iowa.

Das, D., K. Sikdar & A. K. Chetterjee. 1994. Potential of *Azolla pinnata* as biogas generator and as a fish-feed. Indian J. Environm. Health **36:** 186–191.

De Waha Baillonville, T., H. F. Diara, I. Watanabe, P. Berthet & C. Van Hove. 1991. Assessment and attempt to explain the high performance of *Azolla* in subdesertic tropics versus humid tropics. Pl. & Soil **137:** 145–150.

El-Sayed, A. F. M. 1992. Effects of substituting fish meal with *Azolla pinnata* in practical diets for fingerling and adult Nile tilapia, *Oreochromis niloticus* (L.). Aquac. & Fish. Managem. **23:** 167–173.

Fogg, G. E., W. D. P. Stewart, P. Fay & A. E. Walsby. 1973. The blue-green algae. Academic Press, London.

Forni, C., S. Gentili, C. Van Hove & M. Grilli-Caiola. 1990. Isolation and characterization of the bacteria living in the sporocarps of *Azolla filiculoides* Lam. Ann. Microbiol. Enzimol. **40:** 235–244.

Fremy, P. 1930. Les myxophycees de l'Afrique equatoriale francaise. Arch. Bot. **3:** 373–395.

Hall, D. O., S. A. Markov, Y. Watanabe & K. K. Rao. 1995. The potential applications of cyanobacterial photosynthesis for clean technologies. Photosyn. Res. **46:** 159–167.

Hamdi, Y. A. 1982. Application of nitrogen-fixing systems in soil improvement and management. FAO Soils Bull. 49. Food and Agriculture Organization, Rome.

Hardy, R. W. F., R. D. Holsten, E. K. Jackson & R. C. Burns. 1968. The acetylene-ethylene assay for N_2 fixation: Laboratory and field evaluation. Pl. Physiol. **43:** 1185–1207.

Hechler, W. D. & J. O. Dawson. 1995. Factors affecting nitrogen fixation in *Azolla caroliniana*. Trans. Illinois State Acad. Sci. **88:** 97–107.

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

———. 1977. The role of *Anabaena* in the *Azolla–Anabaena* symbiosis. New Phytol. **78:** 611–616.

Hur, J. S. & A. R. Wellburn. 1993. Effects of atmospheric sulfur dioxide on *Azolla* and *Anabaena* symbiosis. Physiol. Pl. (Copenhagen) **88:** 65–72.

——— & ———. 1994a. Effects of atmospheric O_3 on *Azolla–Anabaena* symbiosis. Ann. Bot. **73:** 205–209.

——— & ———. 1994b. Effects of atmospheric NO_2 on *Azolla-Anabaena* symbiosis. Ann. Bot. **73:** 137–141.

Ismail, B. S., T. C. Sew & I. Mushrifah. 1995. Effect of molinate and carbofuran on nitrogen fixation by *Azolla pinnata*. Microbios **82:** 127–134.

Ito, O. & I. Watanabe. 1985. Availability to rice plants of nitrogen fixed by *Azolla*. Soil Sci. Pl. Nutr. **31:** 91–104.

Jain, S. K., P. Vasudevan & N. K. Jha. 1989. Removal of some heavy metals from polluted water by aquatic plants: Studies on duckweed and water velvet. Biol. Wastes **28:** 115–126.

Johnson, G. V., P. A. Mayeux & H. J. Evans. 1966. A cobalt requirement for symbiotic growth of *Azolla filiculoides* in the absence of combined nitrogen. Pl. Physiol. **41:** 852–855.

Joy, P. P. & G. V. Havanagi. 1990. Studies on the integrated use of fertilizer nitrogen, phosphorus and azolla biofertilizer for rice. Mysore J. Agric. Sci. **22:** 436–443.

Kannaiyan, S. 1993. Nitrogen contribution by *Azolla* to rice crop. Proc. Indian Natl. Sci. Acad., B **59:** 309–314.

Kolhe, S. S. & B. N. Mittra. 1990. *Azolla* as an organic source of nitrogen in a rice–wheat cropping system. Trop. Agric. (Trinidad) **67:** 267–269.

Kondo, M., M. Kobayashi & E. Takahashi. 1989. Effect of phosphorus on *Azolla* and its utilization in rice culture in Niger. Pl. & Soil **120:** 165–170.

Krock, T., J. Alkamper & I. Watanabe. 1991. Azolla's contribution to weed control in rice cultivation. Pl. Res. Developm. **34:** 117–125.

Kushari, D. P. 1991. Multiplication of azolla associated with rice. Intl. Rice Res. Newslett. **16:** 18–19.

——— **& I. Watanabe.** 1992. Differential responses of *Azolla* to phosphorus deficiency. II. Screening method under concentration controlled conditions. Soil Sci. Pl. Nutr. **38:** 65–73.

Laurinavichene, T. V., A. F. Yakunin & I. N. Gogotov. 1990. Effect of temperature and photoperiod duration on growth and nitrogen fixation in *Azolla*. Fiziol. Rast. (Moscow) **37:** 457–461.

Lay, W. L., S. N. Huang & C. T. Wang. 1989. Effects of *Azolla* application on growth and yield of rice. Bull. Taichung Distr. Agric. Improvem. Sta., No. **24:** 3–12.

Liu, C. C., W. D. Wei & D. Y. Zheng. 1984. Some advances in *Azolla* research. Page 57 *in* C. Veeder & W. E. Newton (eds.), Advances in nitrogen fixation research. Martinus Nijhoff, The Hague.

Lu, S. Y. 1987. Methods for using *Azolla filiculoides* sporocarps to culture sporophytes in the field. Pages 27–32 *in* International Rice Research Institute, *Azolla* utilization: Proceedings of the Workshop on *Azolla* Use. International Rice Research Institute, Los Banos, Philippines.

Lumpkin, T. A. 1987a. Collection, maintenance, and cultivation of *Azolla*. Pages 55–94 *in* G. H. Elan (ed.), Symbiotic nitrogen fixation technology. Marcel Dekker, New York.

———. 1987b. Environmental requirements for successful *Azolla* growth. Pages 89–97 *in* International Rice Research Institute, *Azolla* utilization: Proceedings of the Workshop on *Azolla* Use. International Rice Research Institute, Los Banos, Philippines.

——— **& D. L. Plucknett.** 1980. *Azolla*: Botany, physiology and use as a green manure. Econ. Bot. **34:** 111–153.

——— **&** ———. 1982. *Azolla* as a green manure: Use and management in crop production. Westview Press, Boulder, Colorado.

Madhusoodanan, P. V. & P. J. Sevichan. 1992. *Azolla microphylla* Kaulfuss: An economically important biofertilizer for paddy fields of Kerala. J. Econ. Taxon. Bot. **16:** 73–76.

Mahapatra, B. S. & G. L. Sharma. 1989. Integrated management of *Sesbania*, *Azolla* and urea nitrogen in lowland rice under a rice–wheat cropping system. J. Agric. Sci. (Cambridge) **113:** 203–206.

Malavolta, E., W. R. Acorsi, A. P. Ruschel, F. J. Krug, L. I. Nakayama & I. Eimori. 1981. Mineral nutrition and N_2-fixation in *Azolla*. Pages 205–211 *in* P. B. Vose & A. P. Ruschel (eds.), Associative N_2-fixation. Vol. 2. CRC Press, Boca Raton, Florida.

Marwaha, T. S., B. V. Singh & S. K. Goyal. 1992. Effect of incorporation of *Azolla* on wheat (*Triticum aestivum* var HD-2329). Acta Bot. Indica **20:** 218–220.

Mian, M. H. & A. K. M. Azmal. 1989. The response of *Azolla pinnata* R. Brown to the split application of phosphorus and the transfer of assimilated phosphorus to flooded rice plants. Pl. & Soil **119:** 211–216.

Misra, S. & B. D. Kaushik. 1989a. Growth promoting substances of cyanobacteria: I. Vitamins and their influence on rice plants. Proc. Indian Natl. Sci. Acad., B **55:** 295–300.

——— **&** ———. 1989b. Growth promoting substances of cyanobacteria: II. Detections of amino acids, sugars and auxins. Proc. Indian Natl. Sci. Acad., B **55:** 499–504.

Mochida, O. 1991. Spread of freshwater *Pomacea* snails (Pilidae, Mollusca) from Argentina to Asia. Micronesica **3:** 51–62.

Moore, A. W. 1969. *Azolla*: Biology and agronomic significance. Bot. Rev. (Lancaster) **37:** 17–34.

Mshigeni, K. E. 1982. Freshwater algal resources of Tanzania: A review and a discussion on their potential for agriculture, food production and other uses. Pages 175–201 *in* H. A. Hoppe & T. Levring (eds.), Algae in pharmaceutical science. De Gruyter, Berlin.

Murayama, N. 1979. The importance of nitrogen for rice production. Pages 5–23 *in* International Rice Research Institute, Nitrogen and rice. International Rice Research Institute, Los Banos, Philippines.

National Academy of Sciences. 1979. Microbial processes: Promising technologies for developing countries. Report of an *ad hoc* panel of the Advisory Committee on Technology Innovation, Board on Science and Technology for International Development, Commission on International Relations. National Academy of Sciences, Washington, DC.

Newton, J. W. 1976. Photoproduction of molecular hydrogen by a plant–algal symbiotic system. Science **191:** 559–561.

Nickell, L. G. 1958. Physiological studies with *Azolla* under aseptic conditions. I. Isolation and preliminary growth studies. Amer. Fern J. **48:** 103–108.

Nik-Khah, A. & M. Motaghi-Talab. 1992. The use of *Azolla* in lactating cows. Iranian J. Agric. Sci. **23:** 47–56.

Pablico, P. P. & K. Moody. 1991. Effect of fentin acetate on wet-seeded rice, *Pistia stratiotes* and *Azolla pinnata*. Crop Protect. **10:** 45–47.

Park, I. H., K. K. Rao & D. O. Hall. 1991. Photoproduction of hydrogen, hydrogen peroxide and ammonia using immobilized cyanobacteria. Intl. J. Hydrogen Energy **16:** 313–318.

Pasternak, P. S., V. G. Mazepa & G. K. Pristupa. 1988. The resistance of tree and shrub species to industrial emissions in the Poles'e region of the Ukraine. Lesnoe-Khozyaistvo **7:** 54–57.

Peoples, M. B., D. F. Herridge & J. K. Ladha. 1995. Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? Pl. & Soil **174:** 3–28.

Peters, G. A. 1975. The *Azolla–Anabaena azollae* relationship III. Studies on metabolic capacities and a further characterization of the symbiont. Arch. Microbiol. **103:** 113–122.

———. 1976. Studies on the *Azolla–Anabaena azollae* symbiosis. Pages 592–610 *in* W. E. Newton & C. J. Nyman (ed.), Proceedings of the First International Symposium on Nitrogen Fixation. Vol. 2. Washington State University Press, Pullman.

———. 1977. The *Azolla–Anabaena azollae* symbiosis. Pages 231–258 *in* A. Hollaender (ed.), Genetic engineering for nitrogen fixation. Plenum Press, New York.

———. 1978. Blue-green algae and algal associations. BioScience **28:** 580–585.

——— **& B. C. Mayne.** 1974a. The *Azolla–Anabaena azollae* relationship. I. Initial characterization of the association. Pl. Physiol. **53:** 813–819.

——— **&** ———. 1974b. The *Azolla–Anabaena azollae* relationship. II. Localization of nitrogen fixing activity as assayed by acetylene reduction. Pl. Physiol. **53:** 820–824.

——— **& S. K. Perkins.** 1993. The *Azolla* and *Anabaena* symbiosis: Endophyte continuity in the *Azolla* life cycle is facilitated by epidermal trichomes: II. Re-establishment of the symbiosis following gametogenesis and embryogenesis. New Phytol. **123:** 65–75.

Rains, D. W. & S. N. Talley. 1979. Use of *Azolla* in North America. Pages 419–431 *in* International Rice Research Institute: Nitrogen and rice. International Rice Research Institute, Los Banos, Philippines.

Rajendran, R. & R. Reuben. 1988. Laboratory evaluation of the water fern, *Azolla pinnata* for mosquito control. J. Biol. Control **2:** 114–116.

——— **&** ———. 1991. Evaluation of the water fern *Azolla microphylla* for mosquito population management in the rice-land agro-ecosystem of south India. Med. Vet. Entomol. **5:** 299–310.

Ram, H., P. Krishna Raja & M. V. S. Naidu. 1994. Effect of *Azolla* on soil properties and yield of mungbean (*Vigna radiata* L.). J. Indian Soc. Soil Sci. **42:** 385–387.

Robins, R. J., D. O. Hall, D. J. Shi, R. J. Turner & M. J. C. Rhodes. 1986. Mucilage acts to adhere cyanobacteria and cultured plant cells to biological and inert surfaces. FEMS Microbiol. Lett. **34:** 155–160.

Rosenani, A. B. & H. A. Chulan. 1992. Availability of nitrogen from nitrogen-15 labeled *Azolla pinnata* and urea to flooded rice. Pl. & Soil **143:** 153–161.

Satapathy, K. B. 1993. Effect of different plant spacing pattern on the growth of *Azolla* and rice. Indian J. Pl. Physiol. **36:** 98–102.

Saunders, R. M. K. & K. Fowler. 1992. A morphological taxonomic revision of *Azolla* Lam. section *Rhizosperma* (Mey.) Mett. (Azollaceae). Bot. J. Linn. Soc. **109:** 329–357.

——— **&** ———. 1993. The supraspecific taxonomy and evolution of the fern genus *Azolla* (Azollaceae). Pl. Syst. Evol. **184:** 175–193.

Saxena, D. K. 1995. Purification efficiency of *Lemna* and *Azolla* for WIMCO effluent. Proc. Natl. Acad. Sci. India, B **65:** 61–65.

Sculthorpe, C. D. 1967. The biology of aquatic vascular plants. Edward Arnold, London.

Shi, D. J. & D. O. Hall. 1988. The *Azolla–Anabaena* association: Historical perspective, symbiosis and energy metabolism. Bot. Rev. (Lancaster) **54:** 353–386.

Silvester, W. B. 1977. Dinitrogen fixation by plant associations excluding legumes. Page 141 *in* R. W. F. Hardy & A. H. Gibson (eds.), A treatise on dinitrogen fixation: Section IV, agronomy and ecology. Wiley-Inter Science, New York.

Singh, A. L. & P. K. Singh. 1990a. Intercropping of *Azolla* biofertilizer with rice at different crop geometry. Trop. Agric. (Trinidad) **67:** 350–354.

——— **&** ———. 1990b. Phosphorus fertilization and the growth and nitrogen fixation of *Azolla* and blue-green algae in rice field. Indian J. Pl. Physiol. **33:** 21–26.

Singh, D. P. & P. K. Singh. 1995. Response of *Azolla caroliniana* and rice to phosphorus enrichment of the *Azolla* inoculum and phosphorus fertilization during intercropping. Exp. Agric. **31:** 21–26.

Singh, P. K., D. P. Singh & R. P. Singh. 1992. Growth, acetylene reduction activity, nitrate uptake and nitrate reductase activity of *Azolla caroliniana* and *Azolla pinnata* at varying nitrate levels. Biochem. Physiol. Pflanzen. **188:** 121–127.

Singh, R. P. & P. K. Singh. 1988. Symbiotic algal nitrogenase activity and heterocyst frequency in seven *Azolla* species after phosphorus fertilization. Hydrobiologia **169:** 131–318.

——— & ———. 1989. Effect of nitrogen fertilizers on nitrogen fixation and heterocyst frequency of cyanobacterium *Anabaena azollae* in 7 species of *Azolla*. Biochem. Physiol. Pflanzen. **185:** 429–433.

Singh, S., R. Prasad, S. K. Goyal, B. V. Singh, T. S. Marwaha & S. N. Sharma. 1992. Effect of *Azolla*, blue-green algae and fertilizer nitrogen on wetland rice (*Oryza sativa*). Indian J. Agron. **37:** 569–571.

Sisworo, E. L., D. L. Eskew, W. H. Sisworo, H. Rasjid, H. Kadarusman, S. Solahuddin & G. Soepardi. 1990. Studies on the availability of *Azolla* nitrogen and urea nitrogen for rice growth using nitrogen-15. Pl. & Soil **128:** 209–220.

Stumpe, J. M. & P. L. G. Vlek. 1991. Acidification induced by different nitrogen sources in columns of selected tropical soils. Soil Sci. Soc. Amer. J. **55:** 145–151.

Sutton, S. D., G. W. Barrett & D. H. Taylor. 1991. Microbial metabolic activities in soils of old-field communities following eleven years of nutrient enrichment. Environm. Pollut. **73:** 1–10.

Tan, B. C., P. Payawal, I. Watanabe, N. Lacdan & C. Ramirez. 1986. Modern taxonomy of *Azolla*: A review. Philipp. Agric. **69:** 491–512.

Teckle-Haimanot, E. V. D. 1995. Comparison of *Azolla mexicana* and N and P fertilization on paddy taro (*Colocasia esculenta*) yield. Trop. Agric. (Trinidad) **72:** 70–72.

Thangaraju, M. & S. Kannaiyan. 1993. Effect of nitrogen fixing water fern *Azolla* and different forms of urea application on the growth, nitrogen uptake and grain yield of rice crop. Acta Agron. Hung. **42:** 69–76.

Tuan, D. T. & R. Q. Thuyet. 1979. Use of *Azolla* in rice production in Vietnam. Pages 395–405 *in* International Rice Research Institute, Nitrogen and rice. International Rice Research Institute, Los Banos, Philippines.

Tung, H. F. & R. C. Shen. 1985. Studies of the *Azolla pinnata–Anabaena azollae* symbiosis: Concurrent growth of *Azolla* with rice. Aquatic Bot. **22:** 145–152.

Vaishampayan, A., Y. R. Reddy, B. D. Singh & R. M. Singh. 1992. Reduced phosphorus requirement of a mutant *Azolla–Anabaena* symbiotic N_2-fixing complex. J. Exp. Bot. **43:** 851–856.

Van Cat, D., I. Watanabe, W. J. Zimmerman, T. A. Lumpkin & T. De Waha Baillonville. 1989. Sexual hybridization among *Azolla* species. Canad. J. Bot. **67:** 3482–3485.

Van Hove, C. 1989. *Azolla* and its multiple uses with emphasis on Africa. Food and Agriculture Organization, Rome.

Venkataraman, G. S. & B. D. Kaushik. 1980. Save on N fertilizers by the use of algae on rice fields. Indian Farm. **30:** 27–30.

Ventura, W. & I. Watanabe. 1993. Green manure production of *Azolla microphylla* and *Sesbania rostrata* and their long-term effects on rice yields and soil fertility. Biol. Fertil. Soils **15:** 241–248.

———, ——— **& G. B. Mascarina.** 1992. Mineralization of *Azolla* nitrogen and its availability to wetland rice: II. Fertilizer effect and nitrogen-15 uptake by rice from different species of *Azolla* with varying nitrogen contents. Soil Sci. Pl. Nutr. **38:** 505–516.

Wagner, G. M. 1983. The ecology and nitrogen-fixing capacity of freshwater blue-green algae in selected localities in Tanzania. M. Sc. thesis, University of Dar es Salaam.

———. 1988. The role of freshwater blue-green algae in biological nitrogen fixation in selected localities in Tanzania. Pages 29–33 *in* J. R. Mainoya (ed.), Proceedings of the Symposium on Two Decades of Science Development in Tanzania, 1965–1985, Dar es Salaam, Tanzania, 2–6 September 1985. University of Dar es Salaam and Tanzania National Scientific Research Council, Dar es Salaam.

———. 1992. Algae in agriculture with special emphasis on their application in rice production. Pages 359–373 *in* K. E. Mshigeni, J. Bolton, A. Critchley & G. Kiangi (eds.), Proceedings of the First International Workshop on Sustainable Seaweed Resource Development in Sub-Saharan Africa, Windhoek, Namibia, 22–29 March 1992.

———. 1996. The *Utricularia*-Cyanophyta and *Azolla–Anabaena* associations: Their ecology, nitrogen fixation rates, and effects as biofertilizers on rice. Ph.D. thesis, University of Dar es Salaam.

Wang, S. M., Q. L. Wang, S. H. Li & J. R. Zhang. 1991. A study of treatment of spring wheat with growth promoting substances from nitrogen-fixing blue-green algae. Acta Hydrobiol. Sinica **15:** 45–52.

Watanabe, I. 1982. *Azolla–Anabaena* symbiosis—Its physiology and use in tropical agriculture. Pages 169–185 *in* Y. R. Dommergues & H. G. Diem (eds.), Microbiology of tropical soils and plant productivity. Martinus Nijhoff/W. Junk, The Hague.

———. 1984. Use of symbiotic and free-living blue-green algae in rice culture. Outlook Agric. **13:** 166–172.

———. 1985. Limiting factors in increasing N_2-fixation in rice fields. Pages 436–453 *in* H. Ssali & S. O. Keya (eds.), Biological nitrogen fixation in Africa: Proceedings of the First Conference of the African Association for Nitrogen Fixation. The Nairobi Rhizobium Microbiological Resources Centre, Nairobi.

——— **& C. C. Liu.** 1992. Improving nitrogen-fixing systems and integrating them into sustainable rice farming. Pl. & Soil **141:** 57–67.

——— **& C. M. Ramirez.** 1984. Relationship between soil phosphorus availability and *Azolla* growth. Soil Sc. Pl. Nutr. **30:** 595–598.

——— **& W. Ventura.** 1992. Long-term effects of azolla and sesbania on rice yield and fertility of tropical wetland rice soil. Pages 331–342 *in* Proceedings of the International Symposium on Paddy Soils, Nanjing, China, 15–19 September 1992. Academia Sinica, Beijing, China.

———**, N. S. Berja & D. C. Del Rosario.** 1980. Growth of *Azolla* in paddy field as affected by phosphorus fertilizer. Soil Sci. Pl. Nutr. **26:** 301–307.

———**, C. R. Espinas, N. S. Berja & B. V. Alimagno.** 1977. Utilization of the *Azolla–Anabaena* complex as a nitrogen fertilizer for rice. Intl. Rice Res. Inst. Res. Pap. Ser. **11:** 1–15.

———**, T. Lapis, R. Oliveros & W. Ventura.** 1988. Improvement of phosphate fertilizer application to *Azolla*. Soil Sci. Pl. Nutr. **34:** 557–569.

Yatazawa, M., N. Tomomatsu, N. Hosoda & K. Nunome. 1980. Nitrogen fixation in *Azolla-Anabaena* symbiosis as affected by mineral nutrient status. Soil Sci. Pl. Nutr. **26:** 415–426.