Symbiosis, 27 (1999) 333–351
Balaban, Philadelphia/Rehovot

Review article.

Azolla Research and Development: Recent Trends and Priorities

ANDRE LEJEUNE[1], ARSENIA CAGAUAN[1,2] and CHARLES VAN HOVE[1*]
[1]*Laboratory of Plant Biology, Faculty of Sciences, Catholic University of Louvain, Place Croix du Sud 5, Bte 14, B-1348 Louvain-la-Neuve, Belgium, Tel. +32-10-473464, Fax. +32-10-473471, E-mail. vanhove@bota.ucl.ac.be;* [2]*Freshwater Aquaculture Center, Central Luzon State University, Munõz, Nueva Ecija 3120, Philippines*

Received May 1, 1999; Accepted August 16, 1999

Abstract

A few years ago we reviewed (Van Hove and Lejeune, 1996) the history of research on *Azolla* and provided a description of the ten useful characteristics attributed to this plant, at least five of which are unquestionable. These include the capacity to fix atmospheric nitrogen, a high productivity, a high protein content, and a depressive influence on both aquatic weeds and NH_3 volatilization. We also discussed the constraints, often overstated, linked to *Azolla* utilization and suggested strategies for helping farmers benefit from *Azolla*. Here we first summarize the major recent basic and applied research trends on the *Azolla-Anabaena* symbiosis. Some development activities are then described which indicate that, despite the declining utilization of *Azolla* as a green manure for rice in China and other South Asian countries, many initiatives aimed at the productive utilization of this plant are being developed in these countries and in other parts of the world. Although often unnoticed in the scientific forums, due to the essentially pragmatic objectives pursued, at least some of these programs seem very promising. Based on this

*The author to whom correspondence should be sent.

Presented at the 8th Congress of the African Association for Biological Nitrogen Fixation, November 23–27, 1998, Cape Town, South Africa

0334-5114/99/$05.50

information we provide a description of what we consider research priorities, and suggestions are presented for creating an African coordinated research and development program which could help African farmers and therefore Africa.

Keywords: *Azolla, Anabaena,* nitrogen fixation, symbiosis, integrated farming systems

1. *Azolla* Research

International research on *Azolla* culminated during the eighties. From 1980 to 1989 the West Africa Rice Development Association (WARDA), in collaboration with the Catholic University of Louvain, conducted a program aimed at evaluating the usefulness of *Azolla* for West African farmers. The conclusions were clearly positive (Anonymous, 1990). Subsequently, for reasons which have been analysed previously (Van Hove and Lejeune, 1996), support, and therefore activities in this field, have decreased considerably. However, some research areas remain active. On the fundamental side these include systematics, morphology, biochemistry and paleobotany and, on the applied side, *Azolla* utilization in integrated farming systems, *Azolla* as a water purifier and the nutritive value of *Azolla*. Two other fields, although not as well documented, merit mention: *Azolla* as a chemical fertilizer saver, and *Azolla* as a pest. Some of the recent scientific literature on these topics is surveyed in this section.

Systematics

As expected, the major recent advances in *Azolla* and *Anabaena azollae* taxonomy originate from molecular techniques. In an extension of earlier studies, Eskew et al. (1993) used DNA amplification fingerprinting (DAF) on DNA extracted from the whole symbiotic system (*Azolla*, *Anabaena* and other associated bacteria). Depending on the primer used, the contribution of the procaryotic DNA in the DNA band pattern obtained after electrophoresis ranged from 0 to 77% and DNA extracted from this whole system was therefore not suitable for *Azolla* taxonomy studies. DNA extracted from *Azolla* roots was therefore used to produce fingerprints of *Azolla* without interference from its symbionts. DAF patterns of *Anabaena* could easily distinguish symbionts from different species of *Azolla* and were used to confirm the maternal inheritance of *Anabaena* in an *Azolla* sexual hybrid.

The preceding study addressed six *Azolla* accessions belonging to *A. caroliniana*, *A. pinnata*, *A. filiculoides*, *A. microphylla* and a sexual hybrid between *A. filiculoides* and *A. microphylla*. In another study, Van Coppenolle et al. (1993) included twenty-five accessions representing all known species and the worldwide distribution of *Azolla*. In this RAPD study, no interference by DNA from the bacteria associated with *Azolla* was detected in the analysis. While this study and the previous one both enabled the identification of the various species and of individual accessions within each species, it also presented phylogenic relationships within the genus *Azolla*. The plants could be separated into three groups: one group included the two varieties of *A. pinnata*; a second group included *A. nilotica*; and a third group included *A. caroliniana, A. mexicana, A. microphylla, A. filiculoides* and *A. rubra*. In this third group, *A. caroliniana, A. mexicana* and *A. microphylla* were clustered together and separated from a second cluster comprised of *A. filiculoides* and *A. rubra*. In another area the potential of using molecular techniques to contribute to the selection of strains has been demonstrated for *Azolla* strains collected in Mexico and in Paraguay (Zimmerman et al., 1993, 1994).

Other studies focused on *Anabaena* rather than *Azolla*. Van Coppenolle et al. (1995) evaluated the genetic diversity of *Anabaena* strains symbiotically associated with *Azolla* and the evolutionary relationships among these symbionts by means of RFLP. Eleven accessions were analysed and the same groupings as the ones detected in their previous study on *Azolla* DNA (Van Coppenolle et al., 1993) were obtained, reinforcing the idea of a co-evolution between *Azolla* and its symbiont already suggested by Caudales et al. (1995) (see below). Kim et al. (1997) simultaneously amplified the intron of a transfer RNA gene from both the fern and its symbiont and confirmed the grouping of *A. caroliniana, A. mexicana* and *A. microphylla*, separated from two accessions of *A. pinnata*. *A. nilotica* constituted a third group. Caudales et al. (1995) based their analysis on the fatty acid composition of *Anabaena* isolated from *Azolla* and found a different clustering. They grouped the symbionts and hence the host ferns *A. caroliniana, A. mexicana, A. microphylla* and *A. filiculoides* (with the latter slightly separated from the three others) separately from the two varieties of *A. pinnata* and from *A. nilotica* and *A. rubra*. This grouping is not consistent with the results obtained by others and it does not coincide with classical systematics of the genus *Azolla* where *A. rubra* is classified in section *Azolla* along with *A. caroliniana, A. mexicana, A. microphylla* and *A. filiculoides*.

In any instance, the notion of co-evolution stemming from molecular studies raises additional questions as to the systematics of the symbiotic cyanobacterium that has always been considered as a unique species, *A. azollae*. It should be noted that even the inclusion of the symbiont in the genus *Anabaena* has been questioned (Plazinski et al., 1990; Komárek and Anagnostidis, 1989).

Morphology/morphogenesis

Although only a limited number of publications have appeared on the morphology and morphogenetic processes in *Azolla* over the past five years, they shed some light on key aspects of *Azolla* biology.

The symbiosis between *Azolla* and *Anabaena* is maintained throughout the sexual development cycle of *Azolla*. Perkins and Peters (1993) used light, transmission and electron microscopy to follow sporocarp development in *A. mexicana* and the mechanism by which sporocarps become inoculated with symbiotic *Anabaena*. They discovered that branched epidermal trichomes, termed sporangial hairs, are associated with sporangia from the onset of their development. They protrude into the apical colony of *Anabaena* and are later displaced ventrally and covered by the developing involucre. In subsequent steps, the *Anabaena* differentiate into akinetes (resting cells). Of note is that the upper lobe of the leaves whose lower lobe has been directed towards sporocarp formation forms modified leaf cavities where the *Anabaena* also differentiate into akinetes rather than into heterocysts. Events leading to the re-establishment of the symbiosis during sexual reproduction were also investigated (Peters and Perkins, 1993). The germination of akinetes is contingent upon embryogenesis, possibly by diffusion of embryo-produced oxygen and water vapour. A cotyledonary leaf fosters the initial association between the embryo and the *Anabaena* cells and surrounds the shoot apex and its developing true leaves. The first four to five true leaves are unlobed and differentiate one or two multibranched trichomes that occur in a cavity-like structure where *Anabaena* appear to degenerate. It is speculated that they might be cannibalised by the fern. It does appear that both the inoculation of the forming sporocarp and of the young sporophyte with *Anabaena* are highly coordinated processes.

Biochemistry

The leaf cavity of *Azolla* hosts the symbiotic *Anabaena azollae* and other bacteria that reside in a mucilage surrounding the cavity. The centre of this leaf cavity is of gaseous nature, and the cavity is surrounded and completely lined by an envelope (Uheda et al., 1995).

de Roissart et al. (1994a,b) showed that the envelope contains only carbon, oxygen and hydrogen. It is resistant to temperatures exceeding 300°C, to the action of several hydrolases and to concentrated acids but it dissolves in hot concentrated alkalies. The molecular C/O ratio is in the range of 4 to 10. These results are consistent with a cuticle-like nature for the envelope and raise the question of how the transfer of metabolites between the plant and the leaf cavity occurs.

Microspectroscopic analysis revealed that nitrogen compounds are abundant in the cytosol and the outer wall layers of terminal cells of *Azolla* branched, pluricellular trichomes that protrude into the leaf cavity. However, they are scarce in the stem cells of these trichomes, suggesting that fixed nitrogen is quickly incorporated into glutamate in the trichomes and transferred to other parts of the plants, including the apices of the stems where the *Anabaena* cells were also found to be rich in nitrogen compounds, although no heterocysts are found there. Nitrogen compounds were present only in small quantities in simple *Azolla* hairs, supporting earlier studies suggesting that they are involved in carbon rather than nitrogen transfer between the plant and the leaf cavity (Albertano et al., 1993). A similar approach was used by Canini et al. (1993) to localize calcium in the symbiotic system.

Finally, the carbohydrate metabolism of the *Azolla-Anabaena* association grown on dinitrogen alone or on dinitrogen and nitrate and of *Anabaena*-free *Azolla* grown on ammonium or nitrate has been compared and the results discussed in terms of relationships between the partners (Kaplan and Peters, 1998).

Paleobotany

The value of fossil *Azolla* as an environmental indicator has long been recognised (Hoffman and Stockey, 1994). The discovery of fossil specimens has allowed the reconstitution of wetland paleoenvironments of the upper Amazon River basin during the Miocene (Hoorn, 1994) and of certain parts of England during the Pleistocene (Gibbard et al., 1996). Similarly, the presence of fossil *Azolla* at certain periods during the last glaciation in Southern Chile has been used by Heusser et al. (1996) to confirm abrupt climatic changes and by Kumaran et al. (1997) to finely date certain sediments in India. The study of Hoffman and Stockey (1994) has focused on evolutionary tendencies among *Salviniaceae* and shown that the vegetative characteristics of modern *Azolla* species were established by the middle Paleocene.

Water purification

The possible use of *Azolla* in water purification has received interest due to the fact that nitrogen availability is often a factor limiting the macrophytes in waste water treatment. Such a limitation does not apply to *Azolla* due to its symbiotic association with a nitrogen fixing bacterium. *Azolla*, known to accumulate phosphorus, is therefore efficient in retrieving phosphates from even a N-deficient medium; it must be noted nevertheless that nitrites and volatile organic acids sometimes present in waste water are very toxic to

Azolla (Kitoh et al., 1993).

The use of *Azolla* in the removal of heavy metals appears more promising. Physiological responses of *A. caroliniana* to chromium (Wilson and Al-Hamdani, 1997) and aluminium (Ayala-Silva and Al-Hamdani, 1997) were investigated with respect to carbohydrate metabolism: both ions caused an increased accumulation of carbohydrates. In their study on the kinetics and the influence of environmental factors on cadmium toxicity, adsorption and intracellular uptake by *A. pinnata*, Gaur and Noraho (1995a,b) and Noraho and Gaur (1996) demonstrated that 90% of the cadmium removed from the medium was adsorbed in cell walls and the rest was located inside the cells. Dead plants accumulated twice as much Cd as living ones. Additional research in this area was carried out by M. Zhao and J.R. Duncan in South Africa. They used dried and milled *A. filiculoides* in batch and column experiments for the removal of chromium (1997a,b) and nickel (1998a,b). Their method appears to be an interesting alternative to conventional, costly methods of treatment of cadmium or nickel contamined effluents that generate large volumes of toxic sludge. The bound metals could be efficiently desorbed and recovered for possible reuse in industrial processes, and the column biomass could be used for up to five cycles with high regeneration efficiencies.

Nutritive value of Azolla

Azolla has long been used by farmers as feed for their animals, mainly in Asia. Scientific literature is abundant in this field, with more than 200 papers dealing with the nutrition of fish, pigs, poultry, rabbits, ruminants and even humans. A survey of the literature on the chemical composition of *Azolla* (Guyaut, Léonard and Peng, unpublished) reveals considerable variation. Some of the variation can be attributed to differences in species or strains of *Azolla* analysed, to the type and extent of standardisation of the analytical procedures used and to the growth conditions (temperature, light, humidity, available nutrients, etc.) and growth phase of *Azolla* at the time of collection. However some values are so completely out of range that experimental errors cannot be excluded. The following general statements come from this survey. *Azolla* can be considered as one of the most nutritive aquatic plants owing to its high crude protein content (generally 20–30% on a dry matter basis) and its generally good amino acids profile; some limited deficiencies in methionine, cysteine and lysine are often observed. Cytoplasmic carbohydrates (soluble sugars and starch) are usually in the range of 7–13% on a dry matter basis. The water content is high (86–95%), and it is often necessary to partially dry *Azolla* prior to its use as feed. The mineral composition is generally well balanced but the total ash content is sometimes excessive due to exogenous mineral components attached to the roots if *Azolla* is not washed. When the *Azolla* population density is high, its fibre content increases considerably and reduces its digestibility. The lipid content is generally low and the carotenoid content high. Concerning specifically poultry feeding, when *Azolla* is incorporated at a 10–20% level in the diet of chickens, the most often cited effects are an increase in the food quantities ingested, an increase in the number of eggs laid and a denser coloration of the egg yolk, an increase in daily weight gain, no change in the digestibility of crude proteins, fats and crude fibers, and an improved economic output. However, contradictory reports also exist for these various parameters. Cagauan and Pullin (1994) reviewed the use of *Azolla* in aquaculture. The authors note the differences in preference of various fish species for various *Azolla* species. The performances of *Oreochromis niloticus*, *Tilapia rendalli* and *Cichlasoma melanurum* in aquaria are negatively correlated with the *Azolla* level in the diet. However, in the field, fresh *Azolla* improves the growth of *O. niloticus* when other suitable foods are also available. The use of *Azoll*a in integrated farming systems (also see section 1.5) appears promising with reports of higher fish yields in rice-*Azolla*-fish systems than in rice-fish systems. Recent data by Léonard et al. (1998) confirm the good digestibility of *A. filiculoides* by *O. aureus* and suggest it could be an interesting nitrogen input in tropical fish pond ecosystems provided other foods are also available to meet the nutrient requirements of tilapia.

Azolla research in rice-based farming systems

A wealth of information exists on the use of *Azolla* as organic fertilizer for rice and there is no doubt at this point of the beneficial effect of *Azolla* in terms of increasing rice grain yield.

Over the last several years, major research has focused on *Azolla* in rice farming integrated with fish culture instead of just rice-*Azolla* culture. The concept of this farming system is the efficient utilization of *Azolla* as nitrogen source for rice and *in situ* natural feed for the fish. Initial researches on rice-fish-*Azolla* farming systems were conducted in Fujian province, China, in the 1980s (Liu, 1987; Anonymous, 1988). China has been reported to have the most successful rice-fish-*Azolla*-production system (Micha, 1995).

Recent reports on rice-fish-*Azolla* farming systems are primarily from research in China and the Philippines (MacKay, 1995; Callo, 1989). These studies revealed that *Azolla* in rice-fish systems increased fish yield. Yields vary depending on the initial fish size and density, fish species, type of fish culture (monoculture, polyculture), length of culture period, survival rate, physical design of rice-fish plots, and management of the growth of *Azolla* intercrop as fresh fish feed. Yields of the herbivorous fish, such as grass carp (*Ctenopharyngodon idella*) in the rice-fish-*Azolla* system are better than those obtained for other fishes but it should be noted that grass carp also ate some of the young rice, resulting in a decreased yield (Chen et al., 1995). Also in China, farm trials of the rice-fish-*Azolla* with a polyculture of various fish species showed cumulative yields as high as 4–10 t fish and 9–12 t rice/ha on a two seasons basis (Liu and Liu, 1995). Such positive results certainly deserve to be confirmed in other ecosystems and research in this area is certainly desirable in Africa.

In the Philippines, early work on rice-fish-*Azolla* farming systems was carried out under the auspices of the National *Azolla* Action Program from 1983 to 1986 (Callo, 1989) and subsequently was supported by the Bureau of Agricultural Research, Department of Agriculture, Philippines during 1993–1994. In recent years, research on new farming systems such as the integration of ducks in rice-fish-*Azolla* systems have been supported at the Freshwater Aquaculture Center, Central Luzon State University, by the Catholic University of Louvain, Belgium, and the Food and Agriculture Organization of the United Nations (Cagauan et al., 1996).

Azolla research in non rice-based farming systems

Azolla as fresh fodder for foraging fish in pond/lake systems has received little attention even if cultivating *Azolla* on a part (generally more or less 50%) of the surface of fish ponds is a traditional method in some parts of China. In a pig-poultry-fish-*Azolla* production system studied by Gavina (1993), *Azolla* was grown in the pond as food for fish and foraging ducks. For ducks, *Azolla* was also used as fresh feed in combination with poultry grower mash, and dried *Azolla* was provided in combination with commercial feed. In the pig-poultry-fish-*Azolla* system, the net yield of Nile tilapia increased from 8 to 11 kg/ha/d; pigs had the heaviest weight when fed with commercial ration or with 20% dried *Azolla* + 80% commercial ration and the mean final weight and weight gain of ducks did not differ between the *Azolla* rations and the commercial ration. In a cage culture in Laguna lake, Nile tilapia fed with fresh *A. pinnata* had a significantly higher weight gain (75 g/fish) after four months than the control (64 g/fish), which received only natural food (Pantastico et al., 1986).

Effect on chemical N-fertilizer loss

The presence of an *Azolla* mat on the surface of a water body has been demonstrated to significantly reduce volatilization of applied N-fertilizers

(Vlek et al., 1992; Vlek et al., 1995). These observations, obtained in controlled conditions, have been confirmed in paddy fields in the framework of a joined FAO/IAEA coordinated research project (Kumarasinghe and Eskew, 1993). The reasons for this effect have not been completely resolved but they clearly involve a depressive effect of *Azolla* on algal photosynthesis in the underlying water and thus a lowering of its pH and therefore reduced NH_3 volatilization. Other effects, including temporary immobilization of chemical-N into *Azolla* are also suspected.

Azolla as a pest

From time to time *Azolla* has been described as a weed, notably in Australia, New Zealand and United States, but its negative impact has never been quantified. It is the general experience of scientists who have been involved in the promotion of *Azolla* for agronomic purposes that the introduction of exotic strains, sometimes at a large scale as in China and Philippines or, more recently in Madagascar, has never created serious environmental problems. However a detrimental proliferation of *A. filiculoides*, introduced 50 years ago, was recently observed in some parts of South Africa and nine negative effects have been described (Hill, 1998). While some of these effects, such as reduced water surface for recreation, deterioration of aquadiversity under a dense *Azolla* mat and clogging of irrigation pumps, are probably undisputable, others, such as increased siltation, reduced water flow in irrigation canals, reduced quality of drinking water and drowning of livestock, require confirmation and quantitation. As for an increased water loss due to evapotranspiration, the only data from the literature (Diara and Van Hove, 1984) suggest the contrary. The same is true for the impact of *Azolla* on diseases. The only experimental evidence bearing on this matter is the often confirmed inhibitory effect of *Azolla* on mosquito proliferation (Rajendran and Reuben, 1991), even if its effective impact is doubtful.

2. *Azolla* Development

Whereas a review of the literature allows a relatively good quantification of research activities, evaluating the effective utilization of *Azolla* at the farm level is more problematic.

Azolla utilization as rice fertilizer in South East Asia is definitely declining. However, a critical analysis of this evolution, presented elsewhere, shows that there is no basis for extrapolation to other regions of the world (Van Hove and Lejeune, 1996). In fact a number of applications are currently being developed in many countries, which are generally not publicized nor quantified.

From personal communications, the authors are aware of present *Azolla* utilization at the farm level in various parts of China, India, Philippines, Colombia, Bolivia and Brazil. In Madagascar a small NGO development project launched in 1991 has resulted in a large scale adoption of diverse cultural practices based on *Azolla*. Thousands of farmers are now firm adepts of these practices and the project is presently expanding with the support of national authorities, the World Bank, FAO and other sources. Similar development projects were initiated a year ago in Burkina Faso and in Mali under the guidance of agronomists who have been trained in Madagascar; preliminary results are encouraging. In Egypt exotic *Azolla* are now widely present in the majority of the irrigated schemes but no systematic training is given to the farmers for an optimal exploitation of this plant. In many other African countries (Benin, Burundi, Chad, Democratic Republic of Congo, Guinea, Guinea-Bissau, Ivory Coast, Rwanda, Senegal, Sierra Leone and Tanzania) some actions have been undertaken in recent years but few data are available.

If this kind of information is surely not what is expected in a scientific paper, it indicates at least that an exhaustive inventory of what is practically realized by practicians is highly desirable.

3. Applied Research Priorities

Knowledge of *Azolla* biology and agronomy has progressed considerably during the past forty years, but serious investigation is still needed to optimize *Azolla* utilization in sustainable agriculture. The following eight research fields seem especially important to the authors.

Selection of strains adapted to various environments

In addition to other, smaller collections (Watanabe and Van Hove, 1996), hundreds of *Azolla* strains from all over the world are presently maintained in the germplasm collection duplicated at the International Rice Research Institute (Philippines) and at the Catholic University of Louvain (Belgium). The characterization of these strains for their ecological requirements, their productivity, their chemical composition, their palatability and their susceptibility to predators is essential for selecting *Azolla* adapted to various environments and to various uses. Up to the present progress has been limited as such characterizations are tedious and require enormous effort.

Clarification of Azolla and Anabaena azollae taxonomy

Azolla taxonomy is presently very confusing and it is difficult to compare results obtained by different scientists. A review of the most controversial group, i.e. the American species (Van Hove and Evrard, in preparation) hopefully will clarify and simplify the situation. At the subspecific level, being able to link agronomically interesting traits to individual genetic markers would be a major breakthrough. While progress has been achieved in fingerprinting some *Azolla* strains, considerable effort is still needed in this area. Finally, the existing physiological, immunological and biochemical evidence shows that the *A. azollae* associated with different *Azolla* species are not identical. A better knowledge of these bacterial partners could allow the creation of new, more efficient associations, through the technique of heterologous associations production (Lin et al., 1989). This approach has produced at least one useful new partnership (Watanabe et al., 1989).

Understanding the sporulation process

Since the pioneer work of Ashton (1977) considerable effort has been expended to identifying the factors inducing the sexual cycle of *Azolla* but they remain an enigma (Marsh et al., 1998). Considering the agronomic usefulness of sporulating strains, especially in areas where conditions do not allow vegetative reproduction year round, and the unsuccessful attempts to obtain information through experimental research, it would perhaps be fruitful to follow another strategy, based on phenology observations in natural conditions. Collecting data from as many parts of the world as possible and correlating them with climatic and other ecological parameters might provide new insights. Controlling the sexual cycle is a prerequisite for the development of selection programs based on genetics.

Control of predators

One of the important factors limiting *Azolla* utilization in South East Asia has been the proliferation of pests. This proliferation is at least partially linked to high atmospheric humidity and high temperature and has devastating effects. Various methods have been described by Chinese scientists for limiting the impact of such predators but they are time consuming and not really satisfactory. Some *Azolla* strains are more resistant to insect attacks than others (Roberts et al., 1998) and screening *Azolla* collections for such resistance would be beneficial.

Quantitative evaluation of the nutritive value of Azolla

As mentioned above considerable data are available on the chemical composition of *Azolla*, wich is an essential but not the sole parameter affecting its nutritive value. More information is needed on the optimal quantity of *Azolla* for incorporation in diets of various animals in conjunction with palatability, the other nutrients locally available and economics.

Future research areas for Azolla in optimizing farming systems

From the research conducted during the past several years, it seems that *Azolla* is a promising crop for integration into rice-fish culture. There should be more research into the utilization of *Azolla* in other farming systems. Some possible applications to be considered in future investigations are as follows:

Regulation of Azolla growth

Azolla growth tends to cover the water surface, resulting in suppression of aquatic weed and algae growth. This attribute is beneficial for the production of rice but not for fish. A complete *Azolla* cover decreases light intensity by about 90%, and thereby reduces phytoplankton production and therefore the amount of dissolved oxygen by more than 50% (Wagner, 1997). This in turn affects the respiration of fish, hence its productivity. Past research showed that the value of *Azolla* as fresh feed for fish production is manifested only in conjunction with natural food in the water as shown for example in the experiment of Pantastico et al. (1986). *Azolla* as *in situ* feed becomes important to fish with good level natural feeding (Cagauan and Pullin, 1994).

The fast multiplication of *Azolla* plants in some bodies of water could pose problems for waterways and navigation. This condition should be taken into account when *Azolla* is used as fresh feed for fish culture in open bodies of waters such as lakes.

Use of Azolla for decreasing eutrophication in integrated livestock/poultry-fish culture

The reported use of *Azolla* for water purification might be exploited in integrated culture systems. One of the characteristics of integrated livestock/poultry-fish culture is the build up of nutrients from the animal manures that leads to eutrophication. Eutrophication of the pond system leads to water quality deterioration, particularly dissolved oxygen that affects fish production. *Azolla* integrated with this system can absorb excess nutrients and thereby decrease eutrophication.

Azolla utilization in other farming systems

Rice-duck farming is a traditional practice in Asia. Ducks are herded in ricefields after harvest and eat the fallen rice grains, aquatic plants, snails and other natural farm food. In addition ducks herded into the ricefields in the interval from after rice transplanting until rice flowering have been reported to control weeds and insects (Furuno, 1996, personal communication; Manda et al., 1993). Ducks also have been found to effectively control the herbivorous golden apple snails (Cagauan et al., 1996). Therefore farming systems that integrate rice, fish, ducks and *Azolla* may offer promise in terms of increasing farm productivity.

Socio-economic studies of Azolla/rice and pond/lake-based farming systems

The applicability of any farming system will depend on acceptability by the end-users who are the farmers. The overall economic profitability of the system is often the judging point to stimulate farmers interest. Farmers attitudes, capacity and support of extension services should also be considered in future studies.

Quantitative evaluation of the effect of Azolla on NH_3 volatilization

It has been demonstrated that the presence of an *Azolla* mat is able to considerably decrease NH_3 losses from fertilized irrigated rice fields (Vlek et al., 1992; Kumarasinghe and Eskew, 1993; Vlek et al., 1995). This means that with increasing amounts of chemical fertilizers, more intercropping *Azolla* (without incorporation into the soil) could be advocated for economical as well a ecological reasons! This potentially very important effect has received little attention. A better quantification of this effect in various field conditions is essential for evaluating the real benefit of such cultural practices.

Azolla control

As mentioned above *Azolla* is considered as a nuisance in some places and aspects of the negative effects attributed to it certainly need consideration. This poses the acute question of how to reconcile the opposing views of those promoting *Azolla* development for agronomic purposes and those promoting its eradication for other reasons. This question must be explored in a large geographic and socio-economic context with the view of making a decision for or against based on such factors.

It is necessary to analyse the situation for a given area but it is as essential to estimate the consequences of a given strategy in a given locality on surrounding areas. If this falls into the realm of international law, it is up to the scientist to propose solutions which reconcile the various positions.

Hill (1998) considers three control techniques: mechanical, chemical, and biological. After rejecting the first two he pleads for a biological control based on the introduction of an exotic weevil, *Stenopelmus rufinasus*, a predator apparently specific for *Azolla*. Beside the fact that one cannot exclude the possibility that in its new habitat this insect could adapt to some other plants, the major risk is its dissemination in other African countries involved with or considering *Azolla* utilization. Should it not be possible to find a method allowing people who wish to destroy *Azolla* to realize their objective without hindering those who want to make profit of *Azolla*? A natural substance which, at extremely low concentrations, inhibits *Azolla* growth by inducing the differentiation of the *Anabaena azollae* vegetative cells into akinetes is currently being studied in the authors' laboratory. If it proves easy to synthesize and highly specific, as suspected from our present results, this substance could be utilized for controlling *Azolla* proliferation when it is undesirable.

4. Coordinated Research and Development Programs

In 1991, after considering the conclusions of the WARDA *Azolla* project (Anonymous, 1990), the Food and Agriculture Organization of the United Nations had planned to organize an African network for *Azolla* development.

The conclusions of the WARDA *Azolla* project were based on results obtained in research stations and had not been tested at the farm level. During this same period the observed decline in *Azolla* utilization in South East Asia gave a negative reputation to *Azolla* in many circles. Decision makers were apparently unaware of the reasons for this decline, which was actually justified by local ecological and socio-economic conditions clearly different from those prevailing in many parts of Africa (Van Hove, 1989; Van Hove and Lejeune, 1996). As a result FAO was not able to convince funding agencies to sustain such a network.

Meanwhile, some positive actions have been launched, especially in Madagascar, to refine efficient strategies for training trainers and farmers and to demonstrate the usefulness of *Azolla* in various real conditions. It is also a matter of fact that the recent economic evolution in some countries tends to encourage alternative solutions to the use of imported chemical fertilizers and that the shortage of feed in many areas justifies the exploitation of non conventional resources. In recent years more and more people in Africa seem interested in the possibility of utilizing *Azolla* as evidenced from the numerous requests for information on its cultivation.

The time seems ripe for coordinating research and, above all, development actions on a wider scale. This could include the creation of an *Azolla* research and development centre based in Africa. Such a centre would be in charge of the maintenance of an *Azolla* germplasm collection, conduct preliminary selection tests on material from this collection, provide *Azolla* strains adapted to the specific needs of potential users, centralize and dispatch information on actions and results obtained by scientists and practicians in Africa as well as in other parts of the world, suggest coordinated research and development programs and organize specific trainings. An AABNF Web site would greatly facilitate such activities, as well as many others concerning biological N-fixation.

Acknowledgements

The authors thank M. Guyaut, V. Léonard and J.L. Peng for their help in reviewing the literature related to *Azolla* composition, B. Lambillotte for skilful typing and editing of the manuscript. A.G. Cagauan is supported by a fellowship of the International Cooperation Secretariat of the Catholic University of Louvain, Belgium.

REFERENCES

Albertano, P., Canini, A., and Grilli Caiola, M. 1993. Sub-cellular distribution of nitrogen compounds in *Azolla* and *Anabaena* by ESI and EELS analysis. *Protoplasma* **173**: 158–169.

Anonymous, 1988. Organic recycling in Asia and the Pacific: the "rice-*Azolla* -fish" system. *RAPA Bulletin* (FAO) **4**: 1–44.

Anonymous, 1990. *Azolla* Project, Final Report, West Africa Rice Development Association, 40 pp.

Ashton, P.J. 1977. Factors affecting the growth and development of *Azolla filiculoides* Lam. In: *Proceedings of the 2nd National Weeds Conference*, Stellenbosch, South Africa, February 1977, pp. 249–268.

Ayala-Silva, T. and Al-Hamdani, S. 1997. Interactive effects of polylactic acid with different aluminium concentrations on growth, pigment concentrations, and carbohydrate accumulation of *Azolla*. *American Fern Journal* **87**: 120–126.

Cagauan, A.G., Van Hove, C., Orden, E., Ramilo, N., and Branckaert, R.D. 1996. Preliminary results of a case study on integrated rice-fish-*Azolla*-ducks farming system in the Philippines. In: *Integrated Systems of Animal Production in the Asian Region*. H. Hayakawa, M. Sasaki, and D. Kimura, eds. Chiba, Japan, pp. 35–61.

Cagauan, A.G. and Pullin, S.V. 1994. *Azolla* in aquaculture: past, present and future. In: *Recent Advances in Aquaculture*, V.J.F. Muir and R.J. Roberts, eds. Blackwell Science Publishers, Oxford, UK, pp. 104–130.

Callo, D.P. 1989. *Azolla* adaptability and utilization on farmers' fields. In: *Azolla: Its Culture, Management and Utilization in the Philippines*. NAAP, Los Banos, Laguna, Philippines, pp. 109–128.

Canini, A., Albertano, P., and Grilli Caiola, M. 1993. Sub-cellular localization of calcium in *Azolla-Anabaena* symbiosis by chlorotetracycline, ESI and EELS. *Botanica Acta* **106**: 146–153.

Caudales, R., Wells, J.M., Antoine, A.D., and Butterfield, J.E. 1995. Fatty acid composition of symbiotic cyanobacteria from different host plant (*Azolla*) species: evidence for coevolution of host and symbiont. *International Journal of Systematic Bacteriology* **45**: 364–370.

Chen, D., Ying, H., and Shui, M. 1995. Rice-*Azolla*-fish in ricefields. In: *Rice-Fish Culture in China*. K.T. MacKay, ed. International Development Research Center (IDRC), Ottawa, Canada, pp. 169–174.

de Roissart, P., Jacques, C., Waterkeyn, L., Berghmans, P., and Van Hove, C. 1994a. First evidence for the cutinic nature of the envelope at the interface of *Azolla* and its endophytes. In: *Nitrogen Fixation with Non-Legumes*. N.A. Hegazi, M. Fayez, and M. Monib, eds. The American University in Cairo Press, Cairo, Egypt, pp. 133–138.

de Roissart, P., Jacques, C., Waterkeyn, L., and Van Hove, C. 1994b. Further morphological and physico-chemical characterization of the interfacial structure between *Azolla* and its endophytes. In: *Proceedings of the 16th International Symposium on Nitrogen Fixation with Non-Legumes*. N.A. Hegazi, M. Fayez, and M. Monib, eds. September 1993, Ismailia, Egypt. The American University in Cairo Press, Cairo, Egypt, p. 21.

Diara, H.F. and Van Hove, C. 1984. *Azolla*, a water saver in irrigated rice fields? In: *Practical Application of Azolla for Rice Production*. W.S. Silver and E.C. Schroder, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 115–118.

Eskew, D.L., Caetano Anolles, G., Bassamb, B.J., and Gresshoff, P.M. 1993. DNA amplification fingerprinting of the *Azolla-Anabaena* symbiosis. *Plant Molecular Biology* **21**: 363–373.

Gaur, J.P. and Noraho, N. 1995a. Role of certain environmental factors on cadmium uptake and toxicity in *Spirodela polyrhiza* (L.) Schleid. and *Azolla pinnata* R. Br. *Biomedical and Environmental Science* **8**: 202–210.

Gaur, J.P. and Noraho, N. 1995b. Adsorption and uptake of cadmium by *Azolla pinnata*: kinetics of inhibition by cations. *Biomedical and Environmental Science* **8**: 149–157.

Gavina, L.D. 1993. Swine-duck-fish-*Azolla* integration in skyponds of La Union, Philippines. In: *Proceedings of the First International Foundation of Sciences (IFS)-National Research Council of the Philippines (NRCP) Seminar*. April 1993. Makati, Metro Manila, Philippines, pp. 182–189.

Gibbard, P.L., Allen, P., Field, M.H., and Hallam, D.F. 1996. Early pleistocene sediments at Great Blakenham, Suffolk, England. *Quaternary Science Reviews* **15**: 413–424.

Heusser, C.J., Denton, G.H., Hauser, A., Andersen, B.G., and Lowell, T.V. 1996. Water fern (*Azolla filiculoides* Lam) in Southern Chile as an index of paleoenvironment during early deglaciation. *Arctic and Alpine Research* **28**: 148–155.

Hill, M. 1998. *Azolla filiculoides* the first step towards biological control. *Plant Protection News* **51**: 1–3.

Hoffman, G.L. and Stockey, R.A. 1994. Sporophytes, megaspores and massulae of *Azolla stanleyi* from the Paleocene, Joffre Bridge locality, Alberta. *Canadian Journal of Botany* **72**: 301–308.

Hoorn, C. 1994. An environmental reconstruction of the paleo-amazon river system (middle late Miocene, NW Amazonia). *Palaeogeography-Palaeoclimatology-Palaeoecology* **112**: 187–238.

Kaplan, D. and Peters, G.A. 1998. The *Azolla-Anabaena azollae* relationship. XIV. Chemical composition of the association and soluble carbohydrates of the association, endophyte-free *Azolla*, and the freshly isolated endophyte. *Symbiosis* **24**: 35–50.

Kim, J.J.H., Krawczyk, K., Lorentz, W.P., and Zimmerman, W.J. 1997. Fingerpinting cyanobionts and hosts of the *Azolla* symbiosis by DNA amplification. *World Journal of Microbiology and Biotechnology* **13**: 97–101.

Kitoh, S., Shiomi, N., and Uheda, E. 1993. The growth and nitrogen fixation of *Azolla filiculoides* Lam. in polluted water. *Aquatic Botany* **46**: 129–139.

Komárek, J. and Anagnostidis, K. 1989. Modern approach to the classification system of cyanophytes. IV. Nostocales. *Arkiv für Hydrobiologie, Suppl. 82, Heft 3, Algological Studies* **56**: 303–345.

Kumaran, K.P.N., Bonde, S.D., and Kanitkar, M. 1997. An aquilapollenites associated palynoflora from Mohgaonkalan and its implication for age and stratigraphic correlation of deccan intertrappean beds. *Current Science* **72**: 8.590–592.

Kumarasinghe, K.S. and Eskew, D.L. 1993. *Isotopic Studies of Azolla and Nitrogen Fertilization of Rice*. Kluwer Academic Press, Dordrecht, The Netherlands, 145 pp.

Léonard, V., Breyne, C., Micha, J. C., and Larondelle, Y. 1998. Digestibility and transit time of *Azolla filiculoides* Lamarck in *Oreochromis aureus* (Steindachner). *Aquaculture Research* **29**: 159–165.

Lin, C., Liu, Z.Z., Zheng, D.Y., Tang, L.F., and Watanabe, I. 1989. Re-establishment of symbiosis to *Anabaena*-free *Azolla*. *Sciences in China* **32**: 551–559.

Liu, C.C. 1987. Re-evaluation of *Azolla* utilization in agricultural production. In: *Azolla Utilization. Proceedings of Workshop on Azolla Use*, March/April 1985, Fuzhou, Fujian, China. International Rice Research Institute, Philippines, pp. 67–76.

Liu, C.C. and Liu, X.S. 1995. A brief introduction on controlling artificial biosphere in paddy field and its new cropping system. In: *The Management of Integrated Freshwater Agro-Piscultural Ecosystems in Tropical Areas*. J.-J. Symoens and J.-C. Micha, eds. Technical Centre for Agricultural and Rural Co-operation (CTA) and Royal Academy of Overseas Sciences, Brussels, Belgium, pp. 343–350.

MacKay, K.T. 1995. *Rice-Fish Culture in China*. International Development Research Centre, Ottawa, Canada, 264 pp.

Manda, M., Uchida, H., Nakagama, A., Matsumoto, S., Shimoshikiryo, K., and Watanabe S. 1993. Effects of aigamo ducks (crossbred of wild and domestic ducks) herding on weeding and pest control in the paddy fields. *Japan Poultry Sciences* **30**: 365–370.

Marsh, B.H., Corbin, J.L., and Peters, G.A. 1998. Changes in soluble amino acid and polyamine composition associated with increasing plant density and the onset of sporulation in *Azolla*. *Symbiosis* **24**: 315–326.

Micha, J.C. 1995. The management of integrated freshwater agro-piscicultural ecosystems in tropical areas: Summary, conclusions and recommendations. In: *The Management of Integrated Freshwater Agro-Piscultural Ecosystems in Tropical Areas*. J.-J. Symoens and J.-C. Micha, eds. Technical Centre for Agricultural and Rural Co-operation (CTA) and Royal Academy of Overseas Sciences, Brussels, Belgium, pp. 573–685.

Noraho, N. and Gaur, J.P. 1996. Cadmium adsorption and intracellular uptake by two macrophytes, *Azolla pinnata* and *Spirodela polyrhiza*. *Archiv für Hydrobiologie* **136**: 135–144.

Pantastico, J.B., Baldia, S.F., and Reyes, D.M. 1986. Tilapia (*T. nilotica*) and Azolla (*A. pinnata*) Cage farming in Laguna Lake. *Fish Research Journal of Philippines* **11**: 21–28.

Perkins, S.K. and Peters, G.A. 1993. The *Azolla-Anabaena* symbiosis: endophyte continuity in the *Azolla* life-cycle is facilitated by epidermal trichomes. I. Partitioning of the endophytic *Anabaena* into developing sporocarps. *New Phytologist* **123**: 53–64.

Peters, G.A. and Perkins, S.K. 1993. The *Azolla-Anabaena* symbiosis: endophyte continuity in the *Azolla* life-cycle is facilitated by epidermal trichomes. II. Re-establisment of the symbiosis following gametogenesis and embryogenesis. *New Phytologist* **123**: 65–75.

Plazinski, J., Zheng, Q., Taylor, R., Croft, L., Rolfe, B.G., and Gunning, B.E.S. 1990. DNA probes show genetic variation in cyanobacterial symbionts of the *Azolla* fern and closer relationship to free-living *Nostoc* strains than to free-living *Anabaena* strains. *Applied and Environmental Microbiology* **56**: 1263–1270.

Rajendran, R. and Reuben, R. 1991. Evaluation of the water fern *Azolla microphylla* for mosquito population management in the rice-land agro-ecosystem of south India. *Medical and Veterinary Entomology* **5**: 299–310.

Roberts, J.M.F., Hance, T., and Van Hove, C. 1998. Fecundity and ovipositional preference of *Elophila Africalis* on *Azolla*: test for host plant susceptibility. *Journal of African Zoology* **112**: 215–222.

Uheda, E., Kitoh, S., Dohmaru, T., and Shiomi, N. 1995. Isolation and analysis of gas bubbles in the cavities of *Azolla* leaves. *Physiologia Plantarum* **93**: 1–4.

Van Coppenolle, B., McCouch, S.R., Watanabe, I., Huang, N., and Van Hove, C. 1995. Genetic diversity and phylogeny analysis of *Anabaena azollae* based on RFLPS detected in *Azolla-Anabaena azollae* DNA complexes using NIF gene probes. *Theoretical and Applied Genetics* **91**: 589–597.

Van Coppenolle, B., Watanabe, I., Van Hove, C., and McCouch, S.R. 1993. Genetic diversity and phylogeny analysis of *Azolla* based on DNA amplification by arbitrary primers. *Genome* **36**: 686–693.

Van Hove, C. 1989. *Azolla* and its multiple uses with emphasis on Africa. FAO, Rome, Italy, 53 pp.

Van Hove, C. and Lejeune, A. 1996. Does *Azolla* have any future in agriculture? In: *Biological Nitrogen Fixation Associated with Rice Production*. M. Rahman, ed. Kluwer Academic Press, Dordrecht, The Netherlands, pp. 83–94.

Vlek, P.L.G., Fugger, W., and Biker, U. 1992. The fate of fertilizer N under *Azolla* in wetland rice. In: 2nd Esa Meeting, Warwick University, UK.

Vlek, P.L.G., Diakite, M.Y., and Mueller, H. 1995. The role of *Azolla* in curbing ammonia volatilization from flooded rice systems. *Fertilizer Research* **42**: 165–174.

Wagner, G.M. 1997. *Azolla*: a review of its biology and utilization. *Botanical Review* **63**: 1–26.

Watanabe, I., Chang, L., Ramirez, C., Lapis, M.T., Santiago Ventura, T., and Liu, Z.Z. 1989. Physiology and agronomy of *Azolla-Anabaena* symbiosis. In: *Nitrogen Fixation With Non Legumes*. F.A.Skinner, ed. Kluwer Academic, Dordrecht, The Netherlands, pp. 57–62.

Watanabe, I. and Van Hove, C. 1996. Phylogenetic, molecular and breeding aspects of *Azolla-Anabaena* symbiosis. In: *Pteridology in Perspective*. J.M. Camus, M. Gibby, and R.J. Johns, eds. Royal Botanic Gardens, Kew, UK, pp. 611–619.

Wilson, G. and Al-Hamdani, S. 1997. Effects of chromium (VI) and humic substances on selected physiological responses of *Azolla caroliniana*. *American Fern Journal* **87**: 17–27.

Zhao, M. and Duncan, J.R. 1997a. Batch removal of sexivalent chromium by *Azolla filiculoides*. *Biotechnology and Applied Biochemistry* **26**: 179–182.

Zhao, M. and Duncan, J.R. 1997b. Column sorption and desorption of hexavalent chromium from aqueous solution and electroplating effluent using *Azolla filiculoides*. *Ressource and Environmental Biotechnology* **2**: 51–64.

Zhao, M. and Duncan, J.R. 1998a. Removal and recovery of nickel from aqueous solution and electroplating rinse effluent using *Azolla filiculoides*. *Process Biochemistry* **33**: 249–255.

Zhao, M. and Duncan, J.R. 1998b. Bed depth service time analysis on column removal of Zn^{2+} using *Azolla filiculoides*. *Biotechnology Letters* **20**: 37–39.

Zimmerman, W.J., Quintero Lizaola, R., and Ferrera-Cerrato, R. 1993. The genetic identification of species of agronomic *Azolla* Lam. indigenous to Mexico. *American Fern Journal* **83**: 97–104.

Zimmerman, W.J., Simon, C.J., Watanabe, I., and Krawczyk, K. 1994. Collection of *Azolla microphylla* in Paraguay and its characterization by allozymes and DNA-RAPDS. *American Fern Journal* **84**: 86–93.