6. *Azolla–Anabaena* symbiosis — its physiology and use in tropical agriculture

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1. Introduction

Azolla is a water fern widely distributed in aquatic habitats like ponds, canals, and paddies in temperate and tropical regions. This plant has been of interest to botanists and Asian agronomists because of its symbiotic association with a N_2-fixing blue-green alga and rapid growth in nitrogen-deficient habitats.

Recently, the interest in this plant–alga association has been renewed by the demand for less fossil energy-dependent agricultural technology.

Reviews on updating information were made by Moore [20], Watanabe [42], and Lumpkin and Plucknett [19]. A bibliographic list was published by the International Rice Research Institute [15].

2. Biology and physiology of Azolla–alga relation

Azolla belongs to the Azollaceae, a heterosporous free-floating fern, and is close to the family Salviniaceae. There are six extant species of *Azolla* (Table 1) and 25 fossil species are recorded [14]. These are divided into two subgenera: *Euazolla,* a New World azolla, and *Rhizosperma.* Species differentiation is based on the morphology of the sexual organ.

The number of septa in the glochidia was used as a taxonomic tool to differentiate *Euazolla*. This criterion was questioned by taxonomists because of variations within a given species [10]. In the subgenus *Rhizosperma,* the glochidia are replaced by a root-like structure emerging from the massulae in the microsporangium. In *A. nilotica*, neither the glochidia nor the root-like structure is present on the massulae (Fig. 1).

Because the sporocarps are usually absent in naturally grown azolla, it is difficult to identify species.

Four species of *Euazolla* originated from the New World, but currently these are widely spread in the temperate regions of Europe and Asia. *A. pinnata* is widely distributed in the subtropical and tropical regions of Asia and has been used for agricultural purposes in Asia. *A. nilotica* is a giant water fern, about 10 cm long, and is distributed in central Africa.

The azolla plant has a branched floating stem that bears alternately arranged overlapping leaves and true roots. Each leaf has two lobes — the ventral or lower

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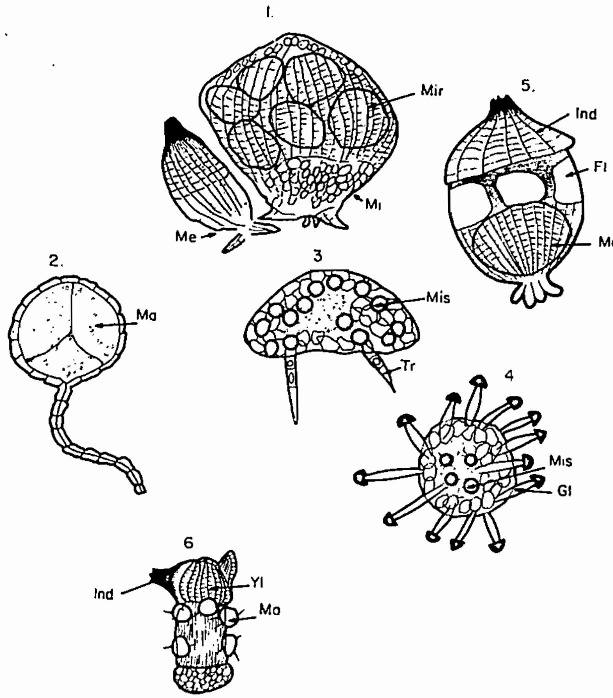

Fig. 1. Sexual organs of *Azolla*.

1. Megasporocarp and Microsporocarp – Me: megasporocarp; Mi: microsporocarp; Mir: microsporangium
2. Microsporangium – Ma: massulae
3. Massulae of *A. pinnata* – Tr: trichome; Mis: microspore
4. Massulae of *A. filiculoides* – Gl: glochidium
5. Megasporocarp – Ind: Indisum; Fl: float; Mes: megaspore
6. Germinating megasporocarp – Yl: young leaf; Ma: massulae

Table 1. Species of *Azolla*

Subgenus	No. of floats on megasporocarps	Species	Major distribution before dispersal by men
Euazolla	3	*A. filiculoides*, Lamarck	Southern South America Western North America
		A, caroliniana, Willd.	Eastern North America Central America
		A. mexicana, Presl.	Northern South America Western North America
		A. microphylla, Kaulfuss	Tropical and subtropical America
Rhizosperma	9	*A. pinnata*, R. Brown	Tropical-subtopical Asia and coastal Africa
		A. nilotica, De Laisne	Upper Nile and Sudan Central Africa

lobe and the dorsal or upper lobe. The dorsal lobes are chlorophyllous and aerial, the ventral lobes are partly submerged, thin, and achlorophyllous. The frond is about 1 to 3 cm long. In optimum condition, the lateral branch of stems of *A. filiculoides* and *A. nilotica* and sometimes, of *A. caroliniana*, partly becomes aerial and new shoots grow upward, thus giving a higher biomass than the flatly growing ones. The roots occur at branch nodes on the ventral surface of the stem. They are about 2 to 10 cm long, depending on species, have hairs and a sheathing root cap that falls off with age. In shallow water, the roots adhere to the soil surface and absorb nutrients from the soil.

In natural conditions, azolla multiply by vegetative reproduction. Under certain circumstances, the formation of sexual organs is observed. A new generation is formed from the fertilized embryo. Although a sporophytic life cycle is described (Fig. 2), little is known about the conditions for spore formation and its ecological significance. High temperature (early summer) in temperate regions and low temperature in tropical and subtropical regions (*A. pinnata*) have been reported to induce sporocarps. In southern China, some local strains of *A. pinnata* form spores abundantly in June and July, and to a lesser extent in September and October. In northern Vietnam, spores are formed in March–April. Formation of sexual organs seems to be associated with high density of azolla population.

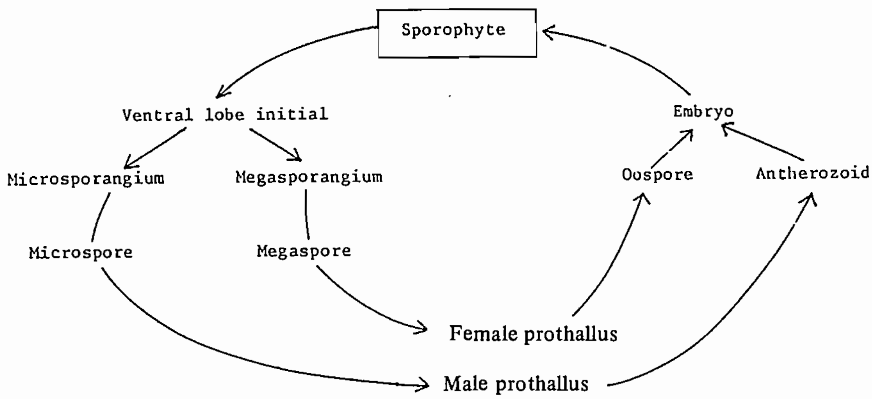

Fig. 2. Heterosporous life cycle of *Azolla*.

When sporocarps are formed, the vegetative growth rate is retarded [2, 3]. Sporocarps are borne by shoot stalks on the first ventral lobe initial of a lateral branch and occur in pairs (Fig. 1).

Microsporocarps, the male organs, are larger than the mega(macro)-sporocarps and are brown yellow or brownish red. They contain many microsporangia and within the periplasmodium of a microsporangium, 32 or 64 microspores develop and aggregate into massulae. From the massulae, glochidia develop. Microspores germinate and release antherozoids which fertilize the oospore in the megaspore.

Megasporocarps, the female organ, are smaller and produce only one megaspore. In mature megasporocarps, the megaspore is covered by 3 or 9 floats together with a columella. One oospore is formed from a megaspore. The germination of embryo and the subsequent growth of young seedling is slow. It takes 1 or 2 months from germination for an azolla to grow as big as a vegetatively growing fern with branches. The germinating megasporocarps are attached by many massulae. The fertilized megaspores can withstand desiccation and can survive more than 1 year in dry condition [46]. Therefore, the fertilized megasporocarps can be used for storage of azolla germplasm collection. Because of the slow growth of a new azolla seedling, the megasporocarps are unlikely to be used as seeding material for agricultural use. Light is necessary for the germination of sporocarps.

The symbiont alga is found in the cavity formed in the proximal portion of the dorsal lobes. The cavity has a mouth which opens toward the ventral side of the dorsal lobes. This mouth probably acts as the site of gas exchange between the atmosphere and the symbiont.

The symbiont alga was named *Anabaena azollae* Strasburger, but Fjerdingstad [7] recently claimed that the alga is actually an ecoform of *Anabaena variabilis*. The isolation of *Anabaena azollae* from azolla has been frequently reported. But none has succeeded in the re-inoculation of the isolated symbiont to alga-free azolla and nothing is known about the free-living state of this symbiont. *Anabaena azollae* was claimed to be associated with the microsporocarps and megasporocarps. It is possible that *Anabaena* in young azolla seedlings originated from the algal cells in megasporocarps.

During the differentiation of dorsal lobe primordia, the cavity occupied by the symbiont is created by epidermal cell growth. Several algal cells sheltered in the shoot apex are entrapped by the enclosing epidermal cells and colonized the cavity [17]. The hair-like cell of plant origin is seen in the youngest cavity. Algal cells in the youngest lobes do not have heterocysts. The frequency of heterocysts increases to about 30% in the 15th leaf as the lobes are traced back from the shoot apex to the basal parts. After the 20th leaf, the heterocysts begin to be senescent [12, 13]. The N_2-fixing ability of each leaf is proportional to the frequency of heterocysts in each leaf.

The frequency of heterocysts is higher in symbiotic alga in azolla than in free-living heterocystous blue-green algae. Hair-like cells are seen in all cavities, which are believed to be the site material exchange between the host plant and the alga [26]. The interaction between both partners is still poorly understood. The growth of azolla and alga may be synchronized. The alga-free azolla has also the cavity and hair-like structure.

The N_2-fixing ability of the symbiont is demonstrated in acetylene reduction assay and $^{15}N_2$. The endophyte algae which are mechanically isolated from the fern have the ability to fix $^{15}N_2$ or to reduce acetylene, although the rate is lower than in association with the fern [23, 24, 25, 27].

The isolated alga excretes about half of the fixed N_2 as ammonium. Because most of the ammonia-assimilating enzyme glutamine synthetase are found in the fern parts of the association, the algae in the association provide fixed N_2 to the host plant mainly as ammonia and the plant converts it to amino acids [25]. N_2 fixation is associated with photosynthesis. Evidences show that the blue-green algae in the association catch solar energy and use this for nitrogenase reaction [28]. It is not known if the energy and reductant necessary for nitrogenase reaction are partly provided by the fern.

The N_2-fixing system in the fern–alga association is not strongly inhibited by ammonium, nitrate, and urea. Azolla growing in 2.5 m*M* ammonium can maintain its growth rate similarly in its absence and N_2 fixation is inhibited by approximately 30%. N_2-fixing activity is quickly recovered upon the transfer to N-free medium (Ito and Peters, unpublished). The N_2-fixing system in the endophyte may be, by some unknown mechanisms, protected from the inhibitory action of combined nitrogen. This is a unique nature of the *Azolla–Anabaena* symbiosis unlike the legume–rhizobium symbiosis which is more sensitive to combined nitrogen.

3. Environmental factors affecting growth and N_2-fixing activities

The growth rate, maximum biomass, and N_2-fixing activity in optimum conditions provide the estimate of the potential of *Azolla–Anabaena* symbiosis for agricultural use.

In optimum light and temperature conditions in the laboratory, Peters *et al.* [29] obtained about 2.0 days doubling time or less for *A. filiculoides, A. caroliniana, A. mexicana,* and *A. pinnata*. Doubling time of 2 days corresponds to 0.34 g^{-1} day^{-1}. Growth in the liquid medium by Talley and Rains [39] with *Azolla filiculoides* and Watanabe *et al.* [42] with *Azolla pinnata* showed that the maximum growth rate was about 2.5 days doubling time.

N_2-fixing rate is estimated by the relative growth rate and nitrogen content of the fern. Assuming 0.277 daily relative growth rate (2.5 days doubling time) and 4% N content in dry matter, daily N_2-fixing rate is calculated as 11.0 mg N g^{-1} dwt. Assuming 4:1 electron ratio of N_2 fixation to acetylene reduction and 12-hr light period a day, this N_2-fixing rate corresponds to 130 μmol C_2H_4 g^{-1} dwt h^{-1}. The reported value of ARA (acetylene reduction activity) fluctuated from 20 to 200 [4].

The growth curve of azolla approximately follows a logistic curve until the biomass reaches the maximum. The growth is characterized by the initial growth rate and the maximum biomass. The growth rate is retarded as the plant density increases [2].

The maximum biomass or nitrogen accumulation reported by researchers is summarized in Table 2. Because *Azolla filiculoides* grows upward from the water surface and forms a thick mat, the maximum biomass is higher than that of other azolla species. No data on the maximum biomass of *A. nilotica*, which forms 10–15 cm long stem, are available. The maximum daily N_2-fixing activity of *A. filiculoides* per unit area was about 2.8 kg N ha^{-1}, whereas the maximum daily N_2-fixing rate of *A. pinnata* grown in open paddy fields in the Philippines was 3.1 kg N ha^{-1} (Watanabe, unpublished). Average daily N_2-fixing rates, measured from

Table 2. Maximum biomass and average N_2-fixing rate

Species	Condition	Maximum biomass			Average N_2-fixing rate (kg ha^{-1} day^{-1})	References
		Dry matter (kg ha^{-1})	N content (kg ha^{-1})	Days		
A. filiculoides	Fallow paddy, USA	1700	52	35	1.5	Talley and Rains [38]
	Shallow pond, USA	1820	105	?		Talley *et al.* [37]
	Paddy soil in pots	5200	128	50	2.6	Tuzimura *et al.* [41]
	Fallow paddy, USA	2300	93	46	2.0	Talley and Rains [39]
A. mexicana	Ponds, USA	830	39	39	1.0	Talley *et al.* [37]
	Paddy field, USA	1100	38	?		Talley and Rains [38]
A. pinnata	Fallow paddy, Philippines	900–1200	48	30–25	1.9–1.6	Watanabe (unpublished)
	Phytotron					
	26° (d)/18° C(n)	2170	96	37	2.6	Watanabe (unpublished)
	33° (d)/25° C(n)	1500	33	22	1.5	Watanabe (unpublished)
	37° (d)/29° C(n)	1120	30	23	1.3	Watanabe (unpublished)
	(var *africana*) greenhouse	640	26	15	1.8	Roger Reynaud [31]
A. filiculoides	26° (d)/18° C(n)	3200	126	51	2.5	Watanabe (unpublished)
A. caroliniana	26° (d)/18° C(n)	3190	146	41	3.6	Watanabe (unpublished)

d = day; n = night

inoculation to harvest, are presented in Table 2. The values fluctuate from 1.0 to 2.6 kg N ha^{-1}. Watanabe *et al.* [44] reported that 26 crops of azolla yielded 450 kg N ha^{-1} for 330 days in an open paddy field. Singh [34] reported an annual production of 333 ton fresh weight ha by weekly harvest and estimated annual nitrogen production at 840 kg N ha^{-1}. Shen *et al.* [33] reported 93–152 kg N ha^{-1} for 45 days.

From these figures, the high potential of azolla as a N_2-fixing crop is easily realized. The fixing rate is almost comparable to the figure of forage legumes [22].

Among environmental factors affecting the growth and N_2-fixing activity of azolla, temperature, light, humidity, and mineral elements are described.

3.1. Temperature

The optimum temperature of *A. pinnata, A. mexicana,* and *A. caroliniana,* when grown in constant temperature under 15 klux artificial light [29] is about 30 °C. *A. filiculoides* requires 25 °C. The response of nitrogenase activity to temperatures ranging from 10 ° to 42 °C also shows that *A. filiculoides* likes lower temperature than *A. pinnata* [4]. Although *A. pinnata* is widely distributed in the tropics, it grows better in cooler seasons. In northern Vietnam, the growth of *A. pinnata* is best in January when the average air temperature is 17 °C. In Varanasi, India, *A. pinnata* grew from July to December but was absent from the ponds [11] in hot summer (April to June). In southern China, azolla grows most abundantly from February to May. In the Philippines, the growth is poorest in April and May when monthly average temperature exceeds 32 °C [44]. Watanabe *et al.* [43] reported that in controlled temperature with 8 °C differences between day and night, the growth of *A. pinnata* in culture solution was about the same at 26 °C (day)/18 °C (night), 29 °C/21 °C, and 32 °C/24 °C, but was reduced by about 50% at 35 °/27 °C (average 31 °C). The maximum biomass is more adversely affected by higher temperature than the growth rate at low plant density [42]. *A pinnata* dies progressively at temperatures higher than 40 °C and lower than 5 °C. Most of the experiments to examine the response of the fern to temperature were conducted at constant day temperature or without the shift of temperature from day to night. *A. filiculoides* could not grow at 40 °C (day)/30 °C (night) temperature. However, if the ferns are grown at lower temperatures and then subjected to a step-wise increase in temperature simulating dawn to midday of the diurnal cycle, the nitrogenase activity increases with temperature up to 40 °C and remains high at 45 °C. Similarly, *A. filiculoides* continues to fix N_2 in the field during hot (40–45 °C) afternoons [39]. Temperature response varies with light intensity [1]. The lower the temperature, the lower the optimum light intensity for the growth of nitrogenase [2, 39]. The temperature response is also dependent on the source of nitrogen [2]. In the tropics, the poor growth of *A. pinnata* in hot summers (average monthly temperature exceeding 30 °C) is a problem to be overcome for the agricultural use of the fern–alga association.

3.2. Light

Short periods of exposure experiments on various light intensities showed that light saturation to nitrogenase is about 250 μE m^{-2} sec^{-1} (20 klux) by Talley and Rains [39], 5 klux by Peters [23] and 10 to 5 klux by Watanabe [42]. For long-term experiments, the fern requires higher light intensity than in the short-term exposure experiments, because the growing fronds overlap each other. Ashton [2] observed that the growth rate of *A. filiculoides* increased with increasing light intensity to a maximum in 50% sunlight (49 klux), but further increase of light intensity retarded the growth rate. Talley and Rains [39] however, did not observe retardation of growth of *A. filiculoides* under artificial light when light intensity was increased from 500 μE m^{-2} sec^{-1} to 1000 (ca. 80 klux) when the temperature during illumination was higher than 25 °C. The apparent discrepancy may be due to the plant density, temperature, and light source. Although shading reduces not only light intensity but also temperature of water and air during sunny midday, experiences tell that shading is beneficial for the growth of *A. pinnata* during hot summer. *A. pinnata, A. mexicana*, and *A. caroliniana* have been observed to turn red in strong sunlight and remain green in shading.

3.3. Humidity

Optimum relative humidity is reported to be 85–90% [47]. At a relative humidity lower than 60%, azolla becomes dry and fragile and more susceptible to adverse condition.

3.4. Mineral requirements

The mineral composition of azolla is summarized in Table 3. Although reported figures vary greatly due to excess uptake, it would be reasonable to assume that macronutrient contents in their sufficiently supplied levels are as follows (in percent to dry weight): N: 4–5, P: 0.5, K: 1.0–2.0, Ca: 0.5, Mg: 0.5, Fe: 0.1.

In batch cultures of azolla in nutrient solution the levels of nutrients to induce mineral deficiency symptoms are reported [41]. Yatazawa *et al.* [45] carefully examined the threshold concentration of macroelements in nutrient solution by using the inoculum that was precultured in the nutrient-deficient solution. Threshold concentrations for *A. pinnata* growth were 0.03, 0.04, 0.4 and 0.5 mmol liter^{-1} for P, K, Mg and Ca, respectively.

Phosphorus-deficient azolla turns reddish brown, fronds become fragile, and roots are elongated and easily detached. *Anabaena* cells become pale green and deformation of vegetative cells and heterocysts occur. The browning of dorsal lobes starts from the newer leaves in *A. mexicana*, but from the older leaves at the basal parts of stems in other species. Reddening of dorsal lobes is more intense in

Table 3. Mineral composition of *Azolla*

Species	Conditions	Percent in dry matter						References
		N	P	K	Ca	Mg	Fe	
A. filiculoides	Water culture	–	0.79	6.5	0.25	0.30	–	Tuzimura *et al.* [41]
A. filiculoides	Soil culture	–	0.95	1.6	1.0	0.36	–	Tuzimura *et al.* [41]
A. filiculoides	Naturally grown	4.5	0.5	2.0	0.97	–	0.1	Buckingham *et al.* [5]
A. pinnata	Naturally grown	4.5	0.5	24.5	0.4	0.5	0.06	Singh [34]
			–0.9		1.0	–0.6	0.26	
A. filiculoides	Naturally grown	2	0.1	2.0	0.6	0.4	0.3	Lumpkin and
		–3.4	–0.4	2.6	–0.8	–0.6	0.5	Plucknett [19]
A. pinnata	Water culture	5.0	1.0	3.2	0.2	0.7	0.08	Watanabe (unpublished)
	(deficient)	–	(0.08)	(0.4)	(0.05)	–	(0.016)	

calcium-deficient azolla, than in phosphorus-deficient plants. The fronds turn fragmented and algal cells are lost from the cavity in the extreme Ca-deficient plant.

In potassium-deficient plants, yellowish browning also occurs. In iron-deficient azolla, chlorophyll content decreases and the plant turns yellowish [43].

Effect of microelements are also reported by Yatazawa *et al.* [45] and Johnson *et al.* [16].

Azolla grows in aquatic habitats and absorbs nutrients mainly from the water. In shallow water the plant root attaches to the soil and plant absorbs nutrients from the soil. Because phosphorus content in soil solution or in paddy water is generally too low to meet the requirement by azolla, the addition of phosphorus is necessary for better growth of azolla [44].

To determine the minimum level of phosphorus in water medium, continuous flow culture was used. At 2 μmol P 1^{-1}, *Azolla pinnata* from Bangkok grew normally, but at 1 μmol P 1^{-1}, the azolla suffered phosphorus-deficiency symptoms [35]. The growth of various species and strains of azolla and the difference in supporting growth at 1 μmol 1^{-1} were observed and compared (Subudhi and Watanabe, unpublished).

4. Agricultural use of Azolla

A book on Chinese agricultural techniques – the essence of feeding people, written in 540 A.D. – describes the cultivation and use of azolla in rice fields. At the beginning of the 17th century (end of Ming dynasty), many local records reported the use of azolla as manure [18].

Azolla used in China originated in Fujiang and Guangdong and spread to rice fields in other provinces, south of the Yantze River. Since the foundation of the People's Republic of China, azolla techniques as green manure for rice and as animal feed have been greatly encouraged and have spread north of the Yantze River. In double cropping of rice in central and southern China, azolla is grown only before the early rice (March–May).

In northern Vietnam, *A. pinnata* has been used as a green manure crop for centuries. It is believed that a peasant in La Van village of Thai Binh province discovered and domesticated the azolla. Before the revolution, some families in the village knew the techniques of rearing azolla starter from April to November. The villagers began selling starter stocks of azolla to regional propagators at high prices [21]. Nowadays, the maintenance and multiplication of starters, and the further propagation from the starters, are systematically conducted and distributed from regional azolla multiplication centers to farmers' cooperatives. Azolla is grown in January and February in paddy fields before spring rice and then incorporated [40].

5. Green manure for rice

Because of its high N_2-fixing ability and nitrogen content, azolla has high potential as green manure crop for wetland rice. It is grown either before or after transplanting rice.

One crop of *A. pinnata* contains about 20–40 kg N ha^{-1}. A Vietnamese study showed that incorporation of 1 ton fresh azolla increased the average rough rice yield by 28 kg in 1958–1967 [6]. If 20 t fresh weight of azolla ha^{-1} is produced before transplanting (this figure is reasonable) about 0.5 t ha^{-1} rice yield increase will be obtained.

In 1975, a conference on azolla for southern China summarized findings from 1,500 experiments in 7 provinces (Chiangxu, Guandong, Fujien, etc). Azolla as manure increased rice yield by 600–750 kg ha^{-1}. Ninety percent of 422 field experiments in Chekiang Province reported an average increase in rice grain yield of 700 kg ha^{-1} or 18.6% [18].

Lately, interest in azolla as green manure for rice was resumed in other south Asian countries. The International Rice Research Institute organized INSFFER (International Network of Soil Fertility and Fertilization Evaluation for Rice) collaborative network activity to test the effect of azolla as green manure. Scientists from 5 countries joined the network and field experiments were conducted at 12 sites in 1979. The results are summarized in Table 4. Positive responses of azolla incorporation either before or after transplanting rice over no nitrogen control were obtained in 10 sites. Growing of azolla before or after the rice produced a rice yield increase equivalent to that obtained from 30 kg N ha^{-1} as urea or ammonium sulphate. Growing of azolla and its incorporation before and after rice increased

Table 4. Effects of azolla and nitrogen fertilizer on rice yield, International Network of Soil Fertility and Fertilizer Evaluation for Rice (INSFFER) trials[a]

Treatment	Average grain yield (t ha^{-1})	Index
1 No nitrogen	2.6	100
2 30 kg N ha^{-1} chemical fertilizer	3.2	122
3 60 kg N ha^{-1}	3.7	141
4 Azolla grown before transplanting, incorporated	3.2	122
5 Azolla grown after transplanting, incorporated	3.1	118
6 Azolla grown after transplanting	3.1	119
7 30 kg N ha^{-1} + Azolla before transplanting, incorporated	3.7	143
8 30 kg N ha^{-1} + Azolla after transplanting, incorporated	3.5	134
9 Azolla grown before and after transplanting, incorporated	3.6	139

[a] Conducted in 8 sites in Thailand, 2 in India, and 1 each in China and Nepal

rice yield equivalent to that obtained from 60 kg N ha^{-1} as chemical N fertilizer. Whether azolla was incorporated or not after it covers fully the paddy surface in the rice canopy did not affect rice yield.

An average N_2-fixing activity of 1–2 kg N ha^{-1} day^{-1}, which is shown by the *Azolla–Anabaena* complex, is sufficient to meet the nitrogen requirement of rice if azolla is grown for the period of one rice cropping.

By widening the distance between rice rows, azolla was grown continuously in the rice canopy [18]. This technique was examined at the International Rice Research Institute. Wide rows (53 cm) were alternated with narrow rows (13 cm). Distance between hills was 6.6 cm. Azolla was grown six or four times and incorporated into the soil after water was drained. A total of 100–70 kg N ha^{-1} were contained in the incorporated azolla. Grain yield was almost equivalent to that obtained from 70–100 kg N ha^{-1} chemical nitrogen fertilizer (Watanabe, unpublished).

The decomposition of azolla is rapid and nitrogen efficiency of azolla is almost comparable or slightly inferior to that of urea or ammonium sulphate [34, 43]. Principally, azolla is grown in the fields, where rice is grown after the azolla harvest or together with azolla as described above. Alternately, azolla is grown continuously year round in paddy fields or in the adjacent ponds. Excess of azolla, after it has been used for rice, can be composted for dryland or vegetable crops.

6. Management practices

The inoculum of azolla must be healthy and fresh. It is continuously multiplied in the inoculum preparation plots or ponds. The inoculum density is an important factor in the efficient production of azolla. Singh [34] recommends 2 tons fresh weight ha^{-1} as the inoculum size. In Vietnam, 5 tons ha^{-1} or more is recommended. When inoculum density is low, azolla is overgrown by algae and weeds.

In Vietnam, the half-saturation method is recommended. The saturated density of *A. pinnata* is about 10–20 t fresh weight ha^{-1}. First, the available inoculum is spread in an area to keep the density at 0.5 kg fresh weight m^{-2}. After one week, the surface is fully covered. Then, half of the azolla is transferred to the open area which has about the size as the area where the azolla was taken (see Fig. 3). After one week or so, both areas are fully covered by azolla. Again, half of the azolla is taken from both areas and transferred to the field about the size of the area where the azolla was taken. By repeating this procedure, the area covered by azolla is exponentially expanded. Azolla is notably responsive to phosphorus fertilizer and requires a continuous supply of water-soluble phosphorus for rapid propagation. Split application of superphosphate is more efficient in promoting azolla growth than basal application and 1 kg P_2O_5 ha^{-1} every 4 days is recommended [44]. The Vietnamese recommended 5–10 kg superphosphate (1–2 kg P_2O_5 ha^{-1}) every 5 days. Singh [34] recommended 4–6 kg P_2O_5 ha^{-1} every week. Superphosphate

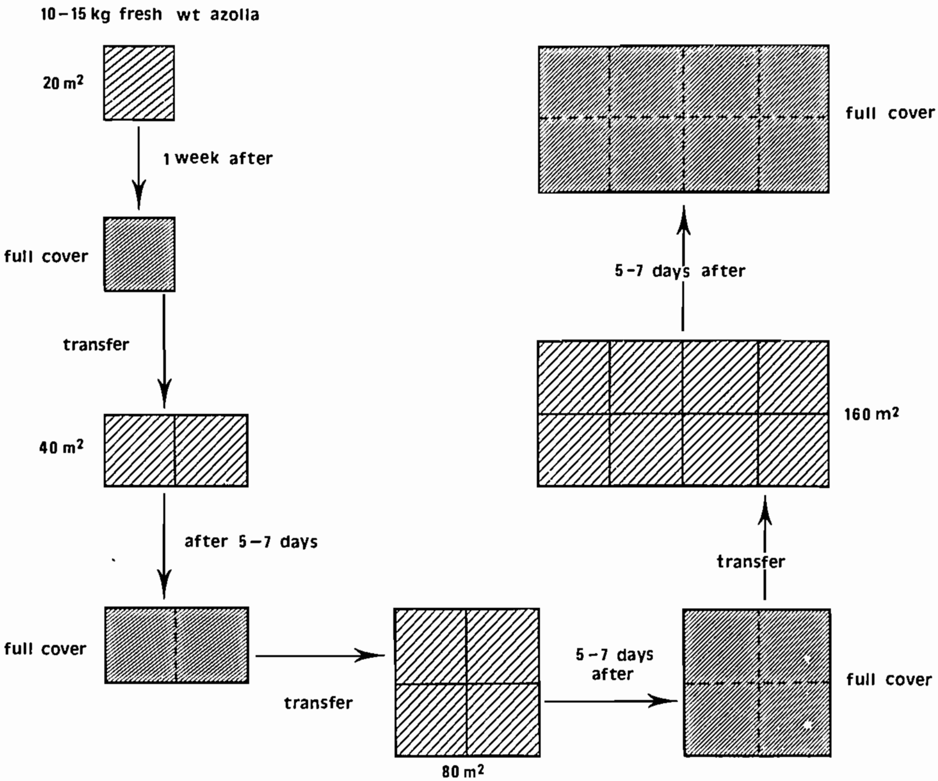

Fig. 3. Schematic of the half-saturation method for *A. pinnata.*

must be applied on the surface of azolla. In China, 3% (w/w) suspension of superphosphate with 0.2 l m^{-2} is recommended.

In a suitable condition each kg of P_2O_5 results in 2 kg additional nitrogen in the azolla biomass [44].

The application of potassium is recommended in light mineral soils. Talley *et al.* [37] obtained significant azolla growth response from a single application of 0.8 kg Fe ha^{-1} as ferric chelate. When soil is flooded before azolla inoculations, iron from the reduced layer becomes available to azolla growth on floodwater.

Water control is critical. Water depth should be kept at about 3–5 cm. When water depth is less than 3 cm, the roots attach to the soil and mineral deficiency is recovered. In hot summer, keeping the water shallow to allow azolla roots to attach to the soil is effective in maintaining healthy azolla. When azolla is incorporated, water is drained and azolla is easily turned under by rake.

Insect damage is serious, particularly in hot summer (higher than 30 °C), because generation time of insects decreases at higher temperature.

Tran and Dao [40] identified the main insect pests of azolla in Vietnam as larvae of *Pyralis, Nymphula,* and *Chironomus* species. In China these genera are also the major and most destructive insect pests [18]. Two species of Nymphula, brown Nymphula (*N. tarbata*) and black Nymphula (*N. swindol*), are important. Adults of brown Nymphula have yellow brown wings and those of black Nymphula have black wings. Their eggs are laid on the back side of ventral lobes and are hatched after 4 or 6 days at 30°C. The larvae attack azolla and form burrows embedded by the silk and fragmented azolla leaves and roots. They come out of the sack while eating azolla. They also eat other aquatic plants.

The gray pyralid-*Pyralis* sp. lays whitish oval eggs on the periphery of the space between the dorsal and ventral lobes. The newly hatched larva is grayish white, but becomes grayish blue or green in later stage. The duration of the 1st and 2nd instar stage at about 30 °C is 3 days. At the end of the 2nd instar, they construct burrows and eat the shoot of the fern. Pupae live for 4–5 days and so do the adults. Total development takes 20 days (Lin Shi-he, Guangxi Province, personal communication). In southern China, this pyralis is the most destructive one. There are 8–10 generations a year, the generations overlap. The damage is easily visible by the lines of burrows. In addition to *Pyralis* and *Nymphula*, Chironomid (*Polypedilium, Microspector, Tendipes,* smout beetle (*Bagous* sp.), and snail (*Radix swinhoer*) are recorded as pests in southern China.

In a hot climate, the damage by fungus attack is also serious, but probably the fungus attack follows the damage by insects or desiccation. *Sclerotium* and *Rhizoctonia* are recorded [32].

Because insect damage is serious in a hot climate (> 30 °C), it is a major constraint in growing azolla in the tropics. A close watch is needed to predict the sudden outbreak of insect pests. The surface of azolla mats should be carefully examined for the presence or traces of insect burrows. The collecting lamp is also useful for the prediction of lepidopterous insects.

The application of 3–4 kg a.i. carbofuran or ferithion per ha is effective, particularly when the application is made with phosphorus fertilizer. Dipping the inoculum in 1000–3000 times diluted insecticide solution is also effective.

In China, bacterial insecticides have also been tried to control azolla pests [18].

7. Other uses of azolla

Azolla can act as a weed suppressor, because the azolla mat covering the surface depresses the growth of weeds.

Rains and Talley [30] reported that early development of *A. filiculoides* eliminated *Cyperus difformus* and *Polygonum* species from the paddy, but not *Echinochloa crusgalli* which is taller. Total biomass was reduced by the azolla cover, although *Echinochloa* predominated. In the International Rice Research Institute's fields where azolla has been continuously grown, sporadic growth of *Monochoria vaginalis* (non-submerged weed) is noticed.

In Hawaii, taro growers used *A. filiculoides* as weed suppressor [8]. But there are opinions that azolla is harmful to rice. Fujiwara *et al.* [9] mentioned that a Japanese farmer who used azolla as green manure observed that a thick mass of azolla covered the young shoot of rice when water was flooded, resulting in the death of rice seedlings. The azolla mat at the early stage of rice, when temperature is still cool, lowers water temperature, resulting in the slight depression of tillering. In direct-seeded rice, quick growth of azolla may be harmful for young rice seedlings.

Azolla has also been used as food to fish and domestic animals in Asia and Africa, because it is rich in protein [36]. It is fed to cattle, pigs, ducks, and fish. Buckingham *et al.* [5] analyzed the nutritional value of *A. filiculoides* and concluded that protein in azolla had a low nutritive value for growing rat. Addition of lysine, methionine, and histidine was effective in improving quality. The high (39%) neutral fibre content of azolla was a major limiting factor in the efficient use of azolla as protein source for monogastric animals. *In vitro* digestability data showed it would be a useful source for ruminants. However, the azolla samples were taken from the creek in an urban area and nothing was described about the age of the fern. Fibre content of their samples was much higher than that reported by Fujiwara *et al.* [9]. Change of nutritive value according to the age of azolla needs to be examined. Singh [34] also suggested the possibility of azolla as human food.

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