USE OF *AZOLLA* AS BIOFERTILIZER IN RICE-WHEAT CROPPING SYSTEM

BY

SIKANDER ALI

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ABSTRACT

Azolla is a free-floating water fern, having a symbiotic nitrogen fixing blue-green alga in its leaves. In rice-*Azolla* culture this symbiotic association can fix nitrogen comparable to legumes. Greenhouse and field studies were conducted to investigate its use as biofertilizer in rice-wheat cropping system being the dominant cropping system in Pakistan.

Before its use in the rice-wheat cropping system, research work was carried out on its natural distribution, selection of heat tolerant strain, cultivation in greenhouse and field conditions, pest control, nitrogen fixation and decomposition, under local conditions.

During the survey of rice growing area of the Punjab, *Azolla pinnata* var. *imbricata* was found growing in stagnant or slow moving waters during the colder months of the year. Since *Azolla* is grown during summer and naturally occurring *Azolla* is sensitive to heat, therefore, through acclimatization processes, a heat tolerant strain was selected for use in greenhouse and field studies.

The analysis of floodwater having good growth of *Azolla* showed that a combination of floodwater properties, like suitable pH, total salt concentration and optimum concentration of essential macro and micronutrients is required for better *Azolla* growth.

The cultivation of *Azolla* in defined culture media, re-synthesized according to floodwater analysis, showed that its growth was better in one of these culture media when adjusted to pH 8, being the common pH of floodwaters of rice area. The addition of humic acid into culture media increased the *Azolla* growth and nitrogenase activity as measured by acetylene reduction assay. In addition to defined culture media, simple and economical undefined culture media were also tested. The addition of farmyard manure (10 g/L), Fe (2 ppm) and superphosphate (10 ppm P) to soil-water culture medium was useful for sustaining the *Azolla* growth for a longer period. Using nutrient missing technique phosphorus was found to be the major limiting nutrient in experimental soils. The application of 10 kg P_2O_5/ha was sufficient to alleviate this constraint.

Amongst the pests, water snails and larvae of Lepidoptera were found to interfere its cultivation. Among the different pesticides tested, Furadan (carbofuran) was found to be the least toxic for *Azolla* growth and was used for pest control.

The effect of combined nitrogen in the culture medium showed that its lower concentration (14 ppm NH_4-N) was useful for its growth while the higher concentrations (28 and 42 ppm) decreasedits growth and nitrogenase activity. The ^{15}N dilution technique was used to estimate nitrogen fixation under field conditions. It was found that average value for nitrogen derived from air for different strains/species was 78%, indicating sufficient nitrogenase activity in the presence of nitrogen under the field conditions.

The decomposition of *Azolla* was investigated by using unlabelled and ^{14}C-labelled *Azolla* by measuring CO_2 evolution. It was found that decomposition rate reached to peak value by 10th day after its incorporation and incubation at 30°C. About three-fourth of the *Azolla*-C was evolved as CO_2 within 2-3 weeks. The decomposition rate was faster in moist soil having 30% saturation percentage (SP) of water than at 100% SP.

The mineralization of *Azolla*-N in soil reached to peak by the end of first week of its incubation and remained almost constant thereafter. The rate of mineralization was faster at 30% than at 100% SP and higher amounts of NO_3-N was formed at 30% SP whereas higher concentration of NH_4-N was observed at 100% SP.

The decomposition of *Azolla* in soil led to formation of humic substances. As estimated by C and N content, about double amount of humic acid and fulvic acid were formed in *Azolla* incorporated soil than control.

The inoculation of *Azolla* resulted into higher leaf area index and higher rice straw and grain yield of a tall variety i.e. Basmati-370 as compared to control and inoculation of blue-green algae. The use of *Azolla* alongwith farmyard manure (FYM) produced equal or more grain yield than urea application at 50 kg N/ha in this rice variety.

The inoculation of *Azolla* also increased the yield of a dwarf rice variety i.e. IR-6. Similarly, *Azolla* cover as well as *Azolla* grown as intercrop and incorporated during rice growth period resulted in more grain yield. The application of *Azolla* was found equivalent to 25-45 kg N/ha of urea. The use of *Azolla* alongwith low amount of urea (30 kg N/ha) was better than urea alone. The use of *Azolla* alongwith urea increased fertilizer-N use efficiency and also reduced N losses from rice ecosystem, due to lowering of floodwater pH. The use of *Azolla* as monocrop indicated that its incorporation first time at 10 and second time at 40 days after transplanting (DAT) during rice growth period was better than its incorporation at 40 and 80 DAT during rice growth. A higher increase in rice yield and more ^{15}N from

Azolla was recovered in rice with early incorporation than late incorporation. The *Azolla* showed a better response for grain yield than urea, whereas chemical-N fertilizer was more useful for increasing straw yield. The application of ^{15}N labelled *Azolla* and urea indicated that a higher amount of ^{15}N was retained in soil for *Azolla* than urea as observed after the harvest of rice.

To investigate the residual effect of *Azolla* on the subsequent crop, wheat was grown without any fertilizer application. The residual effect of *Azolla* alone and alongwith urea was superior to application of urea only. As compared to control, the increase in wheat grain yield was upto 41% for *Azolla* treatments. The residual effect was higher when *Azolla* was incorporated at later stage of rice growth than its incorporation at early stage. Higher amount of ^{15}N was retained in soil after wheat crop for *Azolla* than urea application, indicating its positive role in increasing the N pool of the soil.

The greenhouse and field experiments indicated that *Azolla* can be cultivated as intercrop alongwith rice using the same time and space. Since *Azolla* derived 78% of its nitrogen from air under field conditions, therefore, its use alongwith chemical fertilizer is feasible. The results showed that *Azolla* can effectively be used as a biofertilizer in rice-wheat cropping system under local conditions and a saving of a large amount of chemical N fertilizer and capital is possible.

INTRODUCTION

During the past few decades, there has been much advancement in science and technology leading to greater food production in the world, but the rate at which food production increased in many developing countries has been just sufficient to meet the increased demand for food, resulting from a rapid population growth in these countries. Thus the sufficiency of global food production is still critical and is likely to be more critical in the future (Kumarasinghe and Eskew 1993).

Rice and wheat are the oldest cultivated and most important staple food crops in the world. According to IRRI (1992), in recent years, the rate of yield increase was smaller despite larger input of fertilizer, pesticide and water, and yields of rice-wheat system in South Asia have been steadily declining, with a drop of 10-20%. Therefore, serious efforts are needed to increase the rice and wheat yield, to sustain the productivity and profitability of the rice-wheat rotation for the farmers of Bangladesh, India, Nepal and Pakistan where rice-wheat cropping has increased by ten-fold in the past 30 years. Kundu and Ladha (1995) has also reported a stagnation or even decline in rice yield in some areas of Asia and application of increasing doses of fertilizer N were essential to maintain the original yield level. It was observed that yield declines were commonly associated with decreased uptake of N by the rice crop.

Almost a billion people in South Asia rely on rice and wheat for most of their daily energy (IRRI 1992), while rice is a staple food of over one-half (59%) of the world's population especially in developing countries (IRRI 1994). Therefore, research efforts are required to increase and stabilize the rice and wheat production to avert hunger and malnutrition in the future.

Rice is grown on 148 million hectares, occupying about 10% arable land in the world, and about 90% of this area and 92% of total rice production is in Asia. Although there are about 35 major rice producing countries (17 in Asia, 9 in Latin America, 7 in Africa, and also the USA and Australia), but rice production and consumption are often associated with low income and poverty. About 95% of the world rice is grown in less to least developed countries, primarily in Asia, where population is growing at 2% a year (IRRI 1994) and to match the increasing demand of rice consumption and production, the

rice yield in South and Southeast Asia should increase at least 3% every year (Watanabe 1991). According to another estimate, to feed the increasing global population, the world's annual rice production must increase from the present 520 million tons to 760 million tons by the year 2020 (Kundu and Ladha 1995). The present population of Pakistan is 130 million and is increasing at the rate of 3% per year (Masood 1995), therefore serious efforts are needed to increase the rice and wheat yield being the staple food crops of Pakistan.

In Pakistan rice is grown in more than 2 million hectares, being 10% of the total arable land area of 20.3 million hectares, and about 4.9 million tons (t) of rice is produced in this area per year. Pakistan is the third rice exporting country in the world, exporting 1.2 million t/yr; Thailand being the first, exporting 4.3 million t/year (IRRI 1994). Out of raw item. export of Pakistan of Rs. 30 billion per year, rice earns more than 10 billion rupees, being second to cotton (Rs. 13.4 billion) and thus earns about one-third of this export of Pakistan (MINFA 1992).

The average rice grain yield in Pakistan is 2.4 t/ha, being about two-third of average yield of Asia (3.6 t/ha) and less than half of the maximum yield of 6.2 t/ha obtained by Korea (IRRI 1994). Similarly the average wheat yield/ha in Pakistan is one- third that of Mexico and one-fourth of France (Masood 1995). These figures show that there is ample room for increasing the rice and wheat yield in Pakistan. But nitrogen is the key factor for exploitation of the potential of modern high yielding rice varieties, and in the absence of N input modern varieties yield little more than their traditional counterparts (Brady 1984). Thus the increase in rice and wheat yield will put a heavy burden on the soil resources, and will increase the depletion of nitrogen from the soil if it is not replenished through chemical and/or biological nitrogen fixation (Watanabe 1991).

The large quantities of nitrogen present in the plant, its importance in the structure and metabolism, and the need of the plant for a continuous supply of nitrogen, dramatically point out one of the nature's most paradoxical situation. Since nitrogen composes about 80% of the earth's atmosphere, the plant world may literally be said to be submerged in a sea of nitrogen, yet nitrogen in this form is unavailable to most plants. Indeed, nitrogen is one of the most inert element and requires excessive temperature and pressure in order to react with other elements, as it needs 500° C temperature and 200-500 atmosphere pressure to combine with hydrogen to produce nitrogen fertilizer by Haber Process. The chemical fixation by

Haber Process i.e. the combining of atmospheric dinitrogen with hydrogen to form nitrogen fertilizers, is highly expensive as it uses 2 tons of fuel oil for every ton of nitrogen fertilizer produced (Regan 1988). In Pakistan, nitrogenous fertilizers are produced from natural gas by consuming about 30% of the total gas production of the country. Thus the one-third of the world's agricultural fixed nitrogen (Regan 1988) is not only being produced at a very high cost but is also a big load on the non-renewal energy sources of the world, and also on the countries like Pakistan having limited sources of fossil fuel. Because of the very high price of chemical nitrogen fertilizers, it is unrealistic to advise the farmers of the developing countries to apply fertilizers they could hardly afford, and whose prices are likely to increase in the years ahead (Kumarasinghe and Eskew 1993). In addition to high price, the unwise and excessive use of the nitrogen fertilizer can lead to pollution of ground water. Thus due to high cost of the nitrogen fertilizers, depletion of fossil fuel reserves and problem of pollution, it is very important to find alternatives or supplements to reduce the dependence on chemical N fertilizers, and devise ways and means to exploit the potential of low cost, and safer biofertilizers to increase and sustain crop productivity of our cropping system.

It has been estimated that on global level nearly 200 million ton nitrogen is fixed annually and approximately two-third of it on the earth comes from biological process (Burris 1977). Unlike industrial fixation, the prokaryotic microorganism reduce the elemental nitrogen to ammonia at only 1 atmosphere pressure and at ordinary soil temperature using their nitrogenase enzyme, and the ultimate source for nitrogen fixation is the solar energy. Thus the nitrogen fixation by the microorganism does not put any burden on the fossil fuel and does not create any pollution in the environment. Various nitrogen biofertilizers like *Rhizobium*-legume symbiosis, *Azolla-Anabaena* symbiosis, associative and free living bacteria and blue-green algae have been used for crop production (Burris 1994). The estimated range of nitrogen fixation per crop (and maximum potential) was reported to be 1-7kgN/ha (potential 40 kg) for rice rhizosphere associated bacteria, 10-80 kgN/ha (potential 170 kg) for blue-green algae, 20-150 kgN/ha (potential 224 kg) for *Azolla*, and 20-190 kgN/ha (potential 212 kg) for legumes by Roger and Ladha (1990). These figures show that *Azolla* can provide nitrogen comparable to legumes and much more than free living bacteria and blue-green algae.

The most popular and traditional legume crops are very good as nitrogen biofertilizer especially for upland areas. But they have some weaknesses for rice farmers. One of these weaknesses is that rice is normally grown in fertile soils and farmers are reluctant to use their land, water, and time just for growing a green manure. Secondly, most of the rice fields are flooded and waterlogged during the rice season and most of the legumes can not grow or fix nitrogen in these conditions (Lumpkin and Plucknett 1981). On the contrary, *Azolla* has no such weaknesses rather these conditions are favourable for its growth and nitrogen fixation as this water fern is adapted to aquatic environments, and make it a more suitable nitrogen biofertilizers in rice based cropping system. Thus *Azolla* can be grown in flooded soils before rice as monocrop, or as intercrop with rice using the same land and water at the same time to produce green manure more economically than legumes which require a separate land, time and space for their growth.

The history of *Azolla* use as a green manure in wetland rice in Southeast Asia is very old, and its use has been traced back to around 6th century in China (Liu 1979) and 11th century in Vietnam (Tuan and Thuyet 1979). However, interest in *Azolla* use spread to other countries during 1970's after the availability of information on *Azolla* to other researchers, and its use was studied in different countries (Lumpkin and Plucknett 1982, Roger and Watanabe 1986, Watanabe 1994). The increase in rice yield was reported to be up to 95% in India (Singh 1992), 170% in Thailand (Swatdee et al 1979) and 207% in small plot studies in USA (Talley et al 1977). On the basis of suitability to flooded conditions of rice, ease of incorporation than legumes, positive effect on rice yield and soil fertility, *Azolla* is still considered to be the most suitable nitrogen biofertilizer for rice based cropping system (Roger and Watanabe 1986, Roger et al 1993).

It is reported that *Azolla* can double its biomass in less than two days in laboratory conditions (Peters and Ito 1984) and 3-5 days in favourable field conditions and 5-10 days in normal field conditions (Khan 1983, Watanabe et al 1977). In addition to that it has also been reported that *Azolla* can continue nitrogen fixation even in the presence of combined-N and about 75% of its N was derived from air (Kumarasinghe and Eskew 1993). Thus the ability of *Azolla* to fix nitrogen in the presence of combined nitrogen, makes its use compatible with the present day technology of using chemical N fertilizers for increasing crop production.

To produce 3 tons of rough rice, about 60 kg N should be supplied from soil (Watanabe 1991); while *Azolla*, even when grown as intercrop (dual crop) with rice, can accumulate up to 170 kg N/ha/60 days, exceeding the N requirement of rice (Roger and Watanabe 1986). So in situation, where *Azolla* provides more nitrogen than required by rice, the residual effect on the following wheat crop is expected and a significant increase (85-103%) in subsequent wheat was reported (Kolhe and Mitra 1987).

Considering the long history of *Azolla* use in rice based cropping system, adaptation to rice flooded conditions, growing along with rice as intercrop, more economically than legumes, high rate of growth and nitrogen fixation even in the presence of fertilizer N, positive effect on rice and subsequent wheat yield, its use as biofertilizer was studied in rice-wheat cropping system in Pakistan.

A few years back, when this work was initiated almost no information was available on the distribution, cultivation, nitrogen fixation and its use in cropping system in local conditions of Pakistan. Therefore, the work was carried out on the following aspects:-

Survey of *Azolla*: To know the natural distribution, ecology and for collection of water samples and local *Azolla* strains. survey of the central Punjab, which is the major rice growing area of Pakistan, was carried in different seasons.

Selection of Heat Tolerant Strain and Identification: As rice is grown during summer in Pakistan, thus selection of heat tolerant *Azolla* was necessary for its use in local condition. Therefore, heat tolerant *Azolla* was selected and identified to varietal level, and effect of various temperatures on its growth and sporulation was studied.

Cultivation of *Azolla*: For maintenance of *Azolla* culture in the laboratory or greenhouse, different culture media resembling to local natural habitat were re-synthesized according to floodwater analysis, and were tested and compared for biomass production and nitrogenase activity. For maintenance of *Azolla* nursery in field conditions, simple and undefined culture media were devised and compared for their efficiency. Since successful field cultivation is a prerequisite for its use as a biofertilizer, nutritional constraints were identified and optimum dose of the major limiting nutrient was estimated.

Pest Control and Pesticide Effect on *Azolla*: During *Azolla* cultivation some pests attacked it, therefore different locally available pesticides were tested for their control. After finding

the lethal concentrations of these pesticides on *Azolla* pests, their effect on the *Azolla* growth was also studied.

Nitrogen Fixation, Decomposition and Contribution to Soil Humus: Since information on nitrogen fixation by *Azolla* in the presence of combined N and its mineralization under local conditions was not available, therefore, nitrogen fixation using ^{15}N dilution technique, decomposition of unlabelled and ^{14}C labelled *Azolla*, and its contribution to humic compounds of soil, were investigated under local conditions .

Use of *Azolla* in Rice Crop: To study the effect of *Azolla* on yield of tall and dwarf varieties of rice, different greenhouse and field experiments were carried out. In some of these experiments, ^{15}N labelled *Azolla* was used to trace its N availability to rice, while ^{15}N labelled fertilizer was used to follow up its N uptake in plant and retention in soil, and to compute the fertilizer-N use efficiency.

Residual Effect of *Azolla* on Wheat: Since wheat is grown in most of the paddy area after rice harvest, therefore, residual effect of *Azolla* and chemical fertilizer on the yield of subsequent wheat crop was also studied.

REVIEW OF LITERATURE

THE *AZOLLA* PLANT

Taxonomy:

The genus name, *Azolla*, is a conjugation of two Greek words, Azo (to dry) and ollyo (to kill), suggesting the fern is killed by drought. The genus *Azolla* was established by Lamark in 1783 after examining specimens from Chile; and is grouped with the genus *Salvinia* in the order Salviniales, family Azollaceae, class Filicopsida and division Pteridophyta. As given in Table 1, the genus is divided into two sections (subgenera) primarily on the basis of reproductive organs. In section *Azolla* (formerly *Euazolla*) there are five species; while in *Rhizosperma* only 2 species and one of them i.e. *A. pinnata* is further divided into 2 varieties namely *A. pinnata* var. *pinnata* and *A. pinnata* var. *imbricata* (Lumpkin and Plucknett 1982, Watanabe et al. 1992). Although reproductive characters provide the most useful tool for taxonomic separation (Dunham and Fowler 1987) but in most of the samples the sporocarps are usually absent and hence identification of *Azolla* species is difficult (Lumpkin and Plucknett 1980, Van Hove 1989). The species identification among *A. microphylla, A. mexicana* and *A. caroliniana* is often difficult and controversial (Watanabe et al. 1992) and isozyme technique was also used for their differentiation (Zimmerman et al. 1991).

Geographical Distribution:

The distribution of different *Azolla* species has been described by Olsen (1970), Van Hove (1989) and reviewed by Moore (1969) and Lumpkin and Plucknett (1980, 1982). The native distribution of *Azolla* species has been confirmed through collection or observation of herbarium specimens by Lumpkin (1987). According to him *A. caroliniana* is distributed in the eastern half of the United States, through central America and in South America. *A. filiculoides* is found in Western United States and Canada, through Central America and most of South America and may also be native to Japan.

A. mexicana is found from the west coast of the United States, to Mexico and Central America. The occurrence of *A. microphylla* has been reported in South America, while of

Table 1. Identification of *Azolla* species (Lumpkin and Plucknett 1982, Watanabe et al 1992).

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1. Trichomes present on leaves, as well as on rhizomes. Fronds composed of one or occasionally more main rhizomes with lateral branches. Megaspore with a float. Massula without glochidia or with simple glochidia, branched or unbranched, on portion of massula . . . . . section *Rhizosperma* 2

1. Trichomes only on leaves. Fronds composed of two or more flabelliform main rhizome with lateral branches. Megaspore with 3 floats. Entire massula surface covered with arrow-like glochidia . . . . . section *Azolla* 4

2. Plant somewhat ascending, roots in clusters on the rhizomes nodes, main rhizome up to 40 cm long, sporocarps in clusters of four. Megasporoderm uniformly scrobiculate, dark red with net-like pattern of small spines, hair nearly absent. . . . . *A. nilotica*

2. Uncrowded plants deltoid with one rhizome up to 3 cm long. Megasporoderm with irregular prostrate rods, hair on collar only . . . . . *A. pinnata*

3. Individual plants deltoid or triangular. Main rhizome not dominant but with dichotomous branching . . . . . *A. pinnata* var. *imbricata*

3. Lateral branches regularly pinnate, secondary or tertiary branches scattered along the plant periphery. Main rhizome dominant, plants deltoid or bullet shaped . . . . . *A. pinnata* var. *pinnata*

4. Trichomes single celled, glochidia with few or no septa 5

4. Trichomes 2 or 3 celled, glochidia with several septa 6

5. Trichomes clearly protruding . . . . . *A. filiculoides*

5. Trichomes less protruding. Megaspore similar to *A. filiculoides* but with more numerous and taller pads while collar covered with lesser hair. . . . . *A. rubra*

6. Megasporoderm scrobiculate but with smooth appearance caused by even cover of hair . . . . . *A. microphylla*

6. Megasporoderm with large foveae especially near collar . . . . . .*A. mexicana*

6. Megasporoderm foveae partially masked by a thin weft of hair . . . .*A. caroliniana*

A. nilotica in Africa (Mozambique, Nile River, Sudan, Kenya). *A. pinnata* is found in East and South Asia through equatorial Asia to northern Australia and equatorial and South Africa including Madagascar. *A. rubra (A. japonica)* is found in Japan, Korea, Australia and New Zealand.

Azolla species have been dispersed by man also. *A. filiculoides* and *A. caroliniana* were introduced in Western Europe in the 19th century as an ornamental plant and then it spread to USSR. *A. filiculoides* has also been introduced in the South Africa and China and *A. pinnata* into New Zealand. Different strains of all the species have recently been introduced to research stations of all continents and are being evaluated for field use (Lumpkin 1987). Some of the introduced species were sometimes superior to the indigenous ones, as in Philippines *A. microphylla*, *A. caroliniana* and *A. mexicana* replaced the indigenous *A. pinnata* in different regions. Similarly in northern China *A. filiculoides* introduced from Germany and *A. pinnata* introduced in to Senegal grew much better than the indigenous ones (Watanabe 1994).

Habitat:

Azolla is a delicate free-floating fern, and is commonly found on still water in permanent pools, fresh water ponds, ditches, and paddy fields from temperate to tropical regions. *Azolla* is usually found growing in placid water because it cannot withstand the turbulence and wind associated with vast open water surface or rapidly flowing streams. It may be found in backwaters of sluggish streams and along the banks or even in the centers of lakes and reservoirs among the protective mat of other water plants.

Although *Azolla* is considered a free-floating aquatic plant, it can also grow on damp soils but its growth is slow. American species are more adopted to grow on damp soil due to their vertical growth capability. Since *Azolla* roots can absorb nutrients from soil and sometimes soil can provide nutrients that are deficient in water and the plants growing on damp soil may be healthy and green as compared to red, nutrient-deficient plants growing in nearby water (Lumpkin and Plucknett 1982). Moist cultivation of *Azolla* in China has been reported to give 1/3 to almost equal yield of water cultivation for *A. filiculoides* and *A. caroliniana* species, and was also considered useful for heat tolerance and reducing pest problem (Liu 1987).

Morphology:

The *Azolla* plants are small delicate and moss-like. The free and dense branching results into plants like fronds of a fern (Eames 1936). The *Azolla* plants are triangular or polygonal in shape and float on the surface of water individually or in mats. They give the appearance of dark green to reddish carpet except *A. nilotica* which does not produce the red anthocyanin pigment (Lumpkin and Plucknett 1980). The diploid sporophytes of *Azolla* consist of a horizontal to vertical main rhizome. The stem (rhizome) and its branches are densely clothed with small, alternately arranged, overlapping leaves. The main rhizome, when mature may range in size from 0.5 to 7 cm in diameter (in small species) with 1-5 cm long adventitious roots, while the trailing rhizome of *A. nilotica* may be 15 to 40 cm or more in length with root bundles up to 15 cm or more (Lumpkin 1987b). The roots hang in the water or, when in shallow water, occasionally penetrate into mud and help in uptake of nutrients and in anchoring the plant against drift of winds.

The leaf of *Azolla* consists of two lobes: a thick aerial dorsal lobe and a thin ventral lobe occasionally of a slightly larger size (Fig. 1). The dorsal lobe is chlorophyllous and contains the endophytic blue-green alga within a basal cavity (Peters et al 1980), connected to the atmosphere by a pore on the adaxial side. The surface of the dorsal lobe has an epidermis covered with vertical rows of single-celled stomata and trichomes of one or more cells (Lumpkin and Plucknett 1982). The trichomes of the leaf are useful for identification of species as already mentioned in Table 1.

The thin ventral lobe of the leaf is almost achlorophyllous with few stomata and trichomes and several chambers. The ventral lobe probably helps in floating due to its convex surface touching the water (Eames 1936). It is also said to function in absorption of water and nutrients as *Azolla* plants are known to survive for a small period of time without roots (Lumpkin 1987b).

Azolla - Anabaena Symbiosis:

All dorsal lobes of *Azolla* leaves produce a cavity for housing the *Anabaena* symbiont (Fig. 1). As the leaf primordium differentiate at the growing point, a slight depression is formed near the base on the adaxial side of the dorsal lobes which enlarges to contain a large

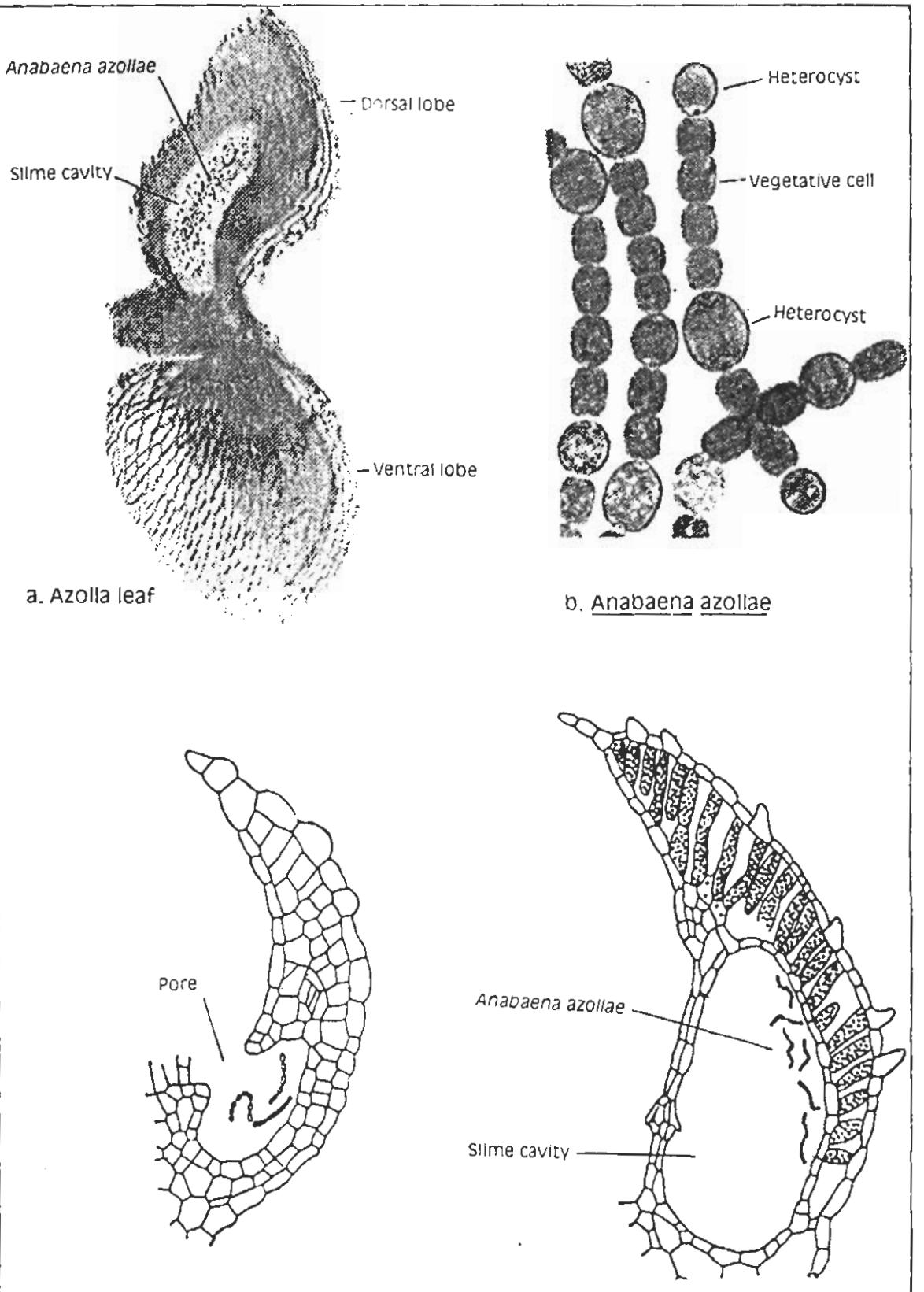

Fig. 1. *Azolla-Anabaena* symbiosis (Sources; a: T.A. Lumpkin 1987, b: G.A. Peters 1977, c&d: G.M. Smith 1955).

number of *Anabaena* filaments, and special branched and simple hair which are involved in the transfer of metabolites between the two partners (Peters et al 1980, Peters et al 1986).

For the development of symbiosis between *Azolla* and *Anabaena,* some physical and chemical processes are involved. There are primary branched hair at the shoot tip and glycoprotein is found on the surface of these hair cells which helps to recognize the *Anabaena* which is always present in association with these multicellular hairs. The undifferentiated *Anabaena* present in close association with these hairs of shoot tip (or under megasporocarp indusium) are considered to be generative in function. A portion of the *Anabaena* colony, generating at the shoot apex above the dorsal lobe primordia, is scooped into the enlarging depression by a hair bridge built between shoot tip and the new cavity by a glove-shaped transfer hair (Zheng 1988). The *Anabaena* becomes entrapped due to the ring of meristematic epidermal cells around the circumference of the depression and grow inwards to cover the depression and leaving a pore over the centre (Fig. 1). The pore may allow the exchange of gases to the endophyte. The inside of the cavity is lined by a porous envelope being cutinic in nature (de Roissart et al 1994) and develops only if the symbiont is present (Peters et al 1978), and the envelope is presumed to help in exchange of metabolites also (Peters et al 1980). After entering the cavity, some vegetative cells of *Anabaena* differentiate into heterocyst (Fig. 1) and exhibit nitrogenase activity (Zheng 1988).

In *A. caroliniana,* the frequency of different cell types in the symbiont, including all stages of leaf development, may be 62% vegetative cells, 21% heterocysts and 17% akinetes (Peters et al 1980). The heterocyst frequency increases from zero at the apical bud to as high as 33% in the 15th leaf and acetylene reduction activity (ARA) is correlated with the increase in heterocyst frequency (Hill 1975, cited by Lumpkin and Plucknett 1982).

In the leaf cavity, housing *Anabaena*, there are two types of special hair, termed simple and branched. The branched hairs are differentiated early during leaf development, there are only two per cavity, always located along the path of the foliar trace and are considered to transfer fixed carbon (sugars, sucrose) from fern to the endophyte. The number of simple hairs increases roughly in parallel with the increase in nitrogenase activity of the endophyte. Each mature cavity contains 20-25 simple hair distributed randomly around the cavity wall adjacent to photosynthetic fern tissue. These simple hair are involved with the assimilation of ammonia (by *Azolla*) released by the endophyte in mature cavity, while

in the early phase of symbiosis, when N_2-fixation has not started in the leaf cavities of apical region, they transfer nitrogen compound(s) from *Azolla* to the *Anabaena* filaments in the apical region (Peters 1981).

The atmospheric N_2 is reduced to NH_3 by the nitrogenase enzyme in the heterocysts, and this newly fixed N is incorporated into glutamine via glutamine synthetase (also present within heterocysts) of *Anabaena azollae* (Ladha and Watanabe 1987). The normal route of nitrogen assimilation in *Anabaena* is by glutamine synthetase and it has very little of this enzyme and about 90% of activity of this enzyme is in the host. Thus the ammonia that *Anabaena* cannot use leaks into to the cavity and is used by the host (Peters et al 1986).

Vegetative Reproduction: Normally *Azolla* reproduces vegetatively. When a frond (*Azolla* plant/sporophyte) reaches a certain size, depending on the species and the environment, generally 1-7 cm in diameter, an abscission layer is formed at the base of old branches (Van Hove et al 1983). The secondary branches extending from the older lateral branches bend the lateral branch and put pressure on its abscission layer, contributing to its separation. These lateral branches then drift away from their parent and become independent plants (Lumpkin and Plucknett 1982). The old Chinese practice of beating *Azolla* with *Azolla* beater, looking like a bamboo broom, for increasing *Azolla* growth in nursery or fields (ZAAS 1975) may be actually helping in separation of *Azolla* at abscission layer leading to faster growth. When *Azolla* is growing on mud or in a crowded mat, fragmentation becomes impossible and results in a decline in the rate of biomass production, therefore exponential growth can only continue under optimum conditions which include adequate space for fragmentation and dispersal of old branches developing into new independent plants (Lumpkin and Plucknett 1982).

Sexual Reproduction: Although *Azolla* normally reproduces vegetatively some species occasionally reproduce through gametophyte cycle (Fig. 2). The gametophytic cycle is usually absent in most situations for most species (Lumpkin 1987). Many species, especially those cultured outside their native habitat, rarely or never become fertile, as one variety of *A. filiculoides* introduced from USA to China by Lumpkin failed to sporulate under a wide range of environmental conditions and only a few species (*A. mexicana*, *A. nilotica* and *A. pinnata*) were consistently fertile in Hawaii (Lumpkin and Plucknett 1982).

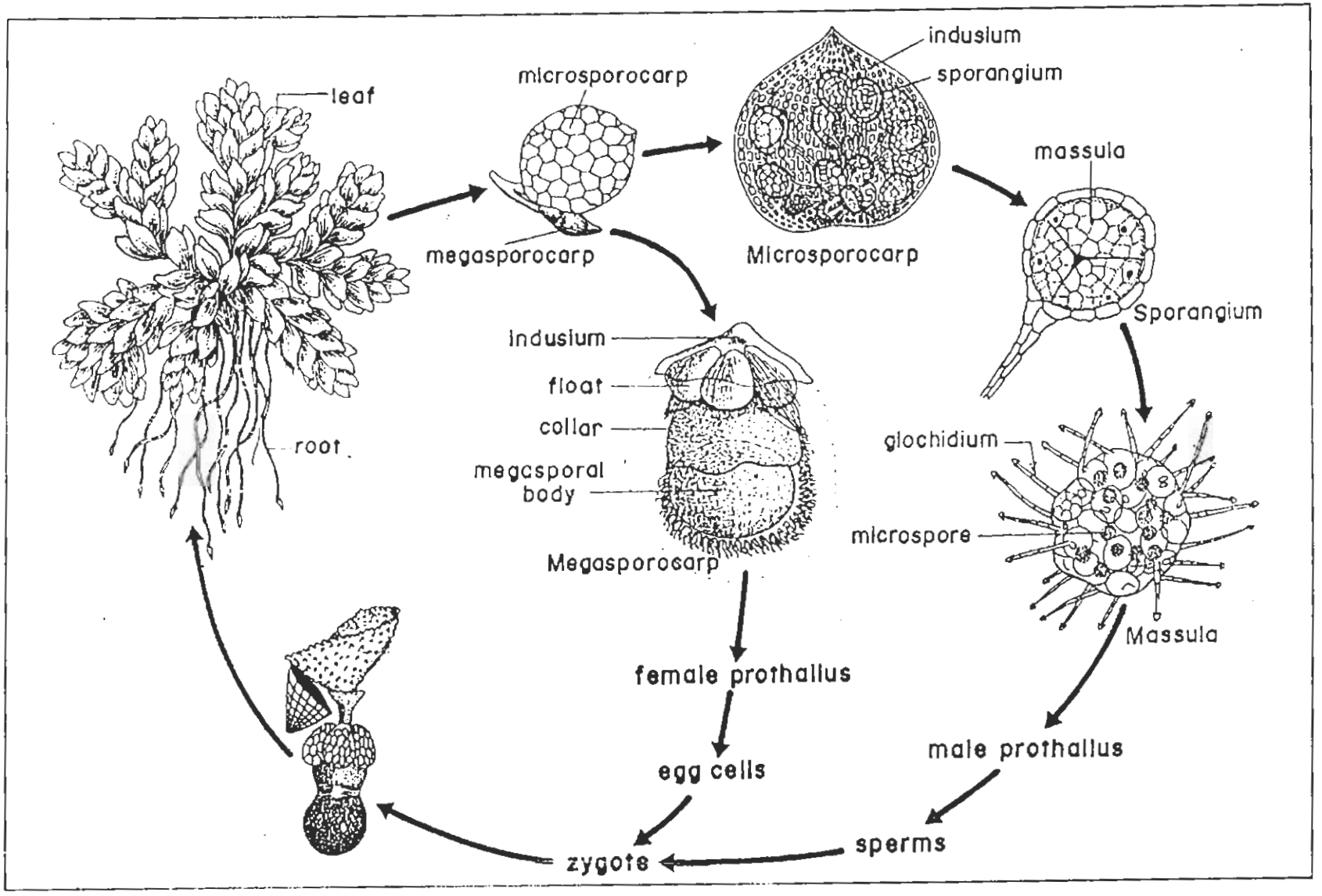

Fig. 2. Sexual reproduction in *Azolla*.

(Source: Watanabe I. 1987. Handout, Int'l.Azolla Training Course: June 6-19 1987, Fuzhou, China)

Initiation of the gametophytic cycle in most species seems to be stimulated by a combination of environmental factors. It was observed that megaspores were formed only during some period of the year i.e. September to January (Ashton 1974). It is also reported that the formation of sporocarp is most often associated with the beginning or end of a period of stress (Lumpkin 1987b). Spores in *A. filiculoides* were produced when the air temperature was 15-25°C (Lumpkin 1985). Environmental factors may also affect the ratio of microsporocarps to megasporocarps; and certain species e.g. *A. filiculoides, A. microphylla* and *A. nilotica* only become fertile after attaining mature morphology, and initiation of this morphology is dependent on environmental conditions (Lumpkin and Plucknett 1982).

When the plants become fertile, sporocarps are formed in pairs (in tetrads on *A. nilotica)* of megasporocarps (macrosporocarp) and microsporocarps (Fig. 2). In case of megasporocarp if a functional megaspore (macrospore) develops in the megasporocarp the remaining 31 megaspore initials or young spores abort (Campbell 1893), but when all the 32 megaspore initials abort then microsporangia initial arise at the base of the same stalk and form sporocarps of one or mixed sexes. Sporocarps mature on the plants in a week or in more time depending on the environmental conditions (Lumpkin and Plucknett 1982).

Megasporocarps may be about 1.5 x 1 mm in *A. filiculoides* (Campbell 1893) but are mostly 0.4-0.6 mm in diameter and have *Anabaena* colony under its dark cone (indusium) resting at its top. The indusium also covers floats being three in section *Azolla* and nine in *Rhizosperma* as already mentioned in Table 1. After maturity, megasporocarp dehisces and exposes sporoderm (perispore) which is usually covered with many hair (Fig. 2) which entangle glochidia of massulae (packets of microspores) except for *A. nilotica* with bald massulae (Lumpkin 1987b). The megaspores of *A. filiculoides* can be induced to germinate in lab at 20°C in a week (Lumpkin 1985), and germination was found to be reduced by exposure to cold (5°C) temperature (Ashton 1974). The megaspore develops into a mature hemispherical mass, 5-8 cells in thickness in about a week (Smith 1955).

Mature microsporocarps are globular in shape, being about 2 mm long and 1.5-1.75 mm in diameter. Each microsporocarp contains up to 32 balloon like microsporangia, and each microsporangium contains 32 or 64 microspores which are packed into 3-10 massulae. After dehiscence of microsporocarp and microsporangial wall, the massulae (packets of

microspores) which may have glochidia (Fig. 2) or may be balded as already mentioned in Table 1, are swept about by water currents and then get attached onto megaspore due to glochidia and minute spines of sporoderm. Microspores germinate and release antherozoids, and fertilize egg in the archegonium developed on the macrogametophyte. The first visible sign of successful fertilization is the tipping of the indusium due to emergence of alga-free cotyledon, and depending upon water temperature, it may take 5 or more days. A portion of the generative *Anabaena* colony surviving under the indusium is trapped into shoot apex and succeeding true leaves. As the root appears and the first or second true leaf emerges through cotyledon, the seedling floats to the surface to begin sporophytic (vegetative) reproduction (Lumpkin and Plucknett 1982).

Sexual Hybridization and Mutation:

Despite the long history of *Azolla* use in Southeast Asia, breeding by sexual hybridization has not been made until recently. In sexual hybridization, when mature megasporocarps are entangled by massulae, the male organs are removed or microsporocarps are removed from the sporophyte to be used as female partner. The female organ of one strain is then mixed with the male organs of the other, and fertilized (sporocarp) sporocarps are grown on N-containing medium until 5 leave stage. As exact time of fertilization is difficult to decide, possibility of self-fertilization always exists (Watanabe 1994), therefore confirmation of hybridization is needed and zymograms has been used for confirmation (Van Cat et al 1989, Zimmerman et al 1991). Most of the hybridization trials resulted in negative heterosis but in some cases positive heterosis was observed and hybrid *Azolla* (*A. microphylla* X *A. filiculoides*) developed at FAAS (China) was found to be better in growth and acetylene reduction activity than the either parent. Similarly, a mutant produced by gamma irradiation at FAAS, has been claimed to be heat and P deficiency tolerant (Watanabe 1994).

ECOPHYSIOLOGY

Like other plants, *Azolla-Anabaena* symbiosis is also affected by environmental factors. The production of anthocyanin, giving the *Azolla* carpet a reddish colour of varying intensities often signals unfavorable conditions such as excessive temperature or light

intensity, or unbalanced nutrition. So, due to diversity of these factors no precise diagnosis can be formulated, however the experienced user may recognize the usual cause of this phenomenon in the local ecological conditions and will react in time to apply appropriate fertilizer or shading to improve the situation (Van Hove 1989). Of the various environmental factors, the availability of water is the most common factor limiting *Azolla* growth. The other important factors are nutrient availability, temperature, light and aspects of water quality such as pH, salinity and turbulence (Becking 1979, Cary and Weerts 1992, Lumpkin and Plucknett 1982, Singh 1992, Watanabe 1991, 1994). In addition to abiotic factors, biotic factors like pests also affect the growth and cultivation of *Azolla* (Lumpkin and Plucknett 1982, Lumpkin 1987, Van Hove 1989).

Mineral nutrition:

The information concerning the mineral requirement of *Azolla* is essential for successful propagation of the fern in the laboratory, green house and under field conditions. *Azolla* like other green plants, requires all the macronutrients (except N) and micronutrients for its growth and nitrogen fixation by its symbiont (Becking 1979, Kitoh and Shiomi 1991, Yatazawa et al 1980). For growing *Azolla* different concentrations of nutrients have been used in 11 different culture media e.g. $H_2PO_4^-$ 0.5-60.0, Ca 1.4-12.0, Mg 1.6-18.8, K 1.3-10.0, Na 0-2.2, SO_4 0.4-24, Cl 0-13, Fe 0.01-3.0 meq/L (Becking 1979). In laboratory cultivation the threshold concentration of P for *Azolla* growth was reported as 0.08 and 0.03, Ca 0.4 and 0.5, Mg 0.3 and 0.4, K 0.3 and 0.4, and S 0.08 m Mol, by Kitoh and Shiomi (1991) and Yatazawa et al (1980) respectively. While the critical concentrations of micronutrients were 50, 20, 0.3 and 30 μg/L for Fe, Mn, Mo and B respectively (Yatazawa et al 1980).

According to Ali and Watanabe (1986) critical concentration of P in *Azolla* was 0.15% on dry weight basis, while in P <0.1 ppm in floodwater caused P deficiency in *Azolla*. P deficient *Azolla* plants develop long profuse and curled roots and may develop red colouration (Lumpkin and Plucknett 1982), and small fronds with yellow green colour and brownish centre were observed by Costa et al (1994). Yatazawa et al (1980) observed that plants deficient in P became slightly pale, and the roots elongated extraordinarily, and necrosis on the fronds was also recognized in plants deficient in K, Ca, or Mg, while plant

colonies deficient in Mg were liable to disintegrate into small colonies. The ferrous ions were reported to be more available than ferric ions at pH 7, and *Azolla* plants became chlorotic with Fe deficiency (Olsen 1970, Yatazawa et al 1980).

Although *Azolla* can grow on purely atmospheric N_2, however if combined nitrogen is available it can also use it, and lower concentrations of nitrate or ammonium may promote its growth (Tuzimura et al 1957). The growth of *Azolla* was found to increase upto 5mM of urea-N, about 3mM NH_4-N, however higher concentrations of combined N decreased its growth in the order of $NH_4 > NO_3 >$ urea (Kitoh and Shiomi 1991). Peters and Ito (1984) found about 60% and 50% of acetylene reduction activity in the presence of 2.5 and 5.0 mM of NH_4-N in the culture medium, while appreciable level of nitrogenase activity in the presence of combined N was also observed by other workers (Becking 1976, Ito and Watanabe 1983, Kitoh and Shiomi 1991, Talley et al 1977 and Watanabe et al 1981).

Azolla grown on very high concentration (20mM) of NH_4-N formed clusters and developed yellow spots, and these yellow spots were found to be *Anabaena*-free (Kitoh and Shiomi 1991).

Water and Humidity:

Water is the most important single factor affecting *Azolla* cultivation. At relative humidity $< 60\%$, *Azolla* becomes dry and fragile while complete drying kills the plant (Becking 1979). The optimum relative humidity is reported to be 85-90%, while very high or very low air moisture were not suitable for *Azolla* growth as at very high humidity the transpiration is low and hence nutrient uptake is hindered (ZAAS 1975).

Azolla multiplies rapidly in free-floating condition than on moist soil, therefore, its cultivation on large scale requires a constant dependable source of water of moderate temperature. Provided that temperatures are not at extreme, a fairly shallow depth of water of 2.5-5 cm is considered most suitable for *Azolla* growth , as in shallow water its roots are in contact with soil (Lumpkin and Plucknett 1982). According to Van Hove (1989) the water strip not more than a few centimeters, not only helps in better nutrition from soil but also reduces the wind effect. According to Singh (1992) 5-10 cm water depth is recommended for good growth, and depth upto 30 cm did not have any adverse effect on *Azolla*. According

to Watanabe (1994), good water control is key for successful *Azolla* cultivation and over-flooding of water may cause spill-over from the desirable site to undesirable places.

Temperature:

After water, temperature is probably the most important environmental factor limiting spread of *Azolla* cultivation, and it is difficult to control also. There are two temperature factors that are important for the growth of *Azolla* i.e. air and water temperature. Of these, air temperature is generally more important during winter, while water temperature during severe summer as at mid-day, temperature of water surface is often 4-7°C higher than the air temperature and may reach 42-45°C at some places. For most widely grown *Azolla* species, the optimum temperature is about 25°C and at this temperature doubling time is 3-5 days and nitrogen fixation capacity is also high, and as the temperature rises to 30°C or above its growth slows down while exposure to temperature much above 40°C for a few hours can kill this plant (Lumpkin and Plucknett 1982, ZAAS 1975). Similarly maximum growth was observed at water temperature of 25°C for two *Azolla* species by Cary and Weerts (1992). The optimum temperature for *A. pinnata* was given as 25-30°C, and although it grew between 14-35°C yet high biomass production and nitrogen fixation was observed during August to October, while minimum during April to June in India (Singh 1992). On the lower end of the scale, temperature below 10°C slowed the growth of *Azolla* and death can occur at temperature of 0°C or below if protective measures are not taken (Lumpkin and Plucknett 1982), and even the cold tolerant varieties of *Azolla* die when the temperature drops below -5°C for more than a few hours (Lumpkin 1987).

The *Azolla* species and varieties differ in their temperature tolerance as *A. filiculoides*, *A. rubra (japonica)* were quite tolerant to low temperature, while *A. mexicana*, *A. microphylla*, *A. caroliniana*, *A. nilotica* and certain varieties of *A. pinnata* possess greater tolerance to high summer temperatures (Lumpkin and Plucknett 1982). During screening for tolerance to high temperature at IRRI, *A. microphylla* from Paraguay and an unclassified *Azolla* from Brazil were found to be most tolerant to high temperature (Watanabe 1991). The extreme temperatures result into reddish brown colouration in *Azolla* (Singh 1992, Van Hove 1989)

Light:

Like other photoautotrophs, light is also essential for *Azolla*. When *Azolla* was grown at 25-30°C and under 400-600 $\mu E/m^2/g$ light conditions, the doubling time of four *Azolla* species was found to decrease from 2-3 days to 1.5-1.7 days with increase in light-dark period from 12-12, to 16-8 and 24-0 (Peters and Ito 1984). Longer days during rice season has also been reported to encourage *Azolla* growth in India (Singh 1992).

The growth of *Azolla* is said to be saturated at approximately 25-50% of full sunlight being 25-50 K lux respectively, and for optimum growth full sunlight was not necessary (Ashton 1974). Optimum light intensity of 500 $\mu E/m/sec$, was observed for growth and nitrogen fixation in *A. filiculides*, for 25/15 to 30/20°C temperature regimes, while at higher temperature (35/25°C) double amount of this light intensity was useful (Talley and Rains 1980), indicating requirement of lower light at lower temperature and higher light intensity for higher temperature. Becking (1979) observed decrease in nitrogenase activity when light intensity reached 80-90 K lux at noon. Cary and Weerts (1992) in Australia found better growth in green house for no shading than shading during summer (Feb) and also in winter (July-August), and adverse effect of shading was less severe during summer with longer day light and higher total radiation than in winter months.

pH:

As regards the quality of water, it should have a suitable pH for growth and nitrogen fixation. The effect of pH on *Azolla* also occur indirectly as it affects the availability of nutrients in floodwater. At very low pH the solubility of Al, Fe and Mn may reach to a toxic level in acid soils and may interfere the absorption of Ca, Mg, and other basic cations, and reduce the solubility of some of the micronutrients like Mo (Lumpkin and Plucknett 1982). In alkaline pH the availability of Ca, Mg and P decreases due to their decreased solubility and most of the micronutrients (except Mo) like Fe, Mn Zn, Cu and B become less available. The availability of P depends on pH and $H_2PO_4^-$ ion is more available than HPO_4^{2-} ions, thus relatively more available phosphate ions are predominant between pH 4.5 to 7.5, and P fixation occurs with Fe, Al, Mn and Mg at lower pH while at higher pH mostly with calcium (Brady 1984).

The optimum pH for *Azolla* growth is reported to be 4.5-7 (Ashton 1974, Carry and Weerts 1992, Watanabe et al 1977), 5-8 according to Singh (1992), however it can survive within a wide range of pH being 3.5-10 (Ashton 1974, Watanabe et al 1977, Singh 1992).

Salinity:

Amount of total salts in water may affect *Azolla* growth, however healthy *Azolla* at 30 different sites was observed in waters having 70-3080 µmho/cm (Lumpkin and Plucknett 1982). The optimum salt concentration for *Azolla* is reported to be 90-150 mg/L, while salinity and alkalinity problems were found in coastal regions and poorly drained soils (Singh 1992). Van Hove et al 1983 reported that 1g NaCl/L was toxic for *Azolla* growth. A decrease in growth and nitrogen fixation in *A. pinnata* due to increase in EC of culture medium from 0.75 to 5.0 dS/m was observed in green house conditions (Ali et al 1990). A decrease in nitrogen fixation in some species of *Azolla* due to addition of NaCl was also observed by Kannaiyan (1992).

Turbulence:

Wave action and turbulence are deleterious to *Azolla*, therefore strong winds can be a problem in *Azolla* cultivation (Lumpkin and Plucknett 1982). The wind accumulates *Azolla* to one side of the stretch of water, creating a premature overcrowding conditions and thus slowing down its growth (Van Hove 1989).

The shaking of *Azolla* culture indicated that its growth and nitrogen fixation was higher at 5 than at 75 revolution per minute (Ashton 1974). The transportation may affect *Azolla* growth, as a lower ARA was found immediately after its transfer, than in *Azolla* plants left undisturbed in the test vessel for 12-14 hours (Becking 1979).

Pests:

According to Hill (1975) "any animal (or plant) which harms or causes damage to man, his animals, crops or possessions or even just causes him annoyance, qualifies for the term pest". From crop point of view, weeds are also pests, and cereals will be pest in a cotton field. Pest status reaches where there is 5% loss in the yield in a particular crop. Thus

during *Azolla* cultivation, other plants and insects or animals may be acting as pests and hindering its growth.

Azolla is a pioneer in areas disturbed by man or animals, where its rapid growth rate and nitrogen fixing ability give it a competitive edge. It can grow in conjunction or succession with the free-floating aquatic plants like *Lemna, Pistia, Salvinia, Spirodela, Ricciocarpus, Riccia* and *Wolfia*. It can grow on damp soil, but it is a poor competitor against terrestrial weeds and eventually succumbs to their pressure. In aquatic conditions sometimes algae entangle to *Azolla* roots and compete for nutrition and space (Lumpkin and Plucknett 1982). The algae may be removed manually or eliminated by drying the fields for a few days or by application of 10 kg copper sulphate/ha (Singh 1992)

Although ferns are one of the most pest resistant groups in the plant kingdom, but *Azolla* is an exception to this generalization and it has wide array of pests ranging from man, animals, fish, insects, fungi, bacteria to viruses. However, out of these, insects, snails and fungi have been reported to affect *Azolla* growth seriously (Lumpkin and Plucknett 1982). In China larvae of Grey Borer, Black Borer, Weevil, Chironomus and Water snails (*Lymnaeidus*) have been reported to eat *Azolla* and animals like frogs, ants, aquatic spiders, parasitic bee, some *Bacillus* and other bacterial preparations were found suitable for biological control of pests (ZAAS 1975). Insects of order Lepidoptera i.e. larvae of *Pyralis* sp. *Nymphula* sp. moth of *Cryptoblabes* and *Samea* sp. while some larvea of Diptera and Coleoptera and water snails were reported to feed on *Azolla* from different countries (Lumpkin and Plucknett 1982, Lumpkin 1987a, Van Hove 1989).

Different insecticides and molluscides were claimed to control the above mentioned insects and snails, while chemicals like Ceresan, Manzate 200 fungicide were found effective against fungal attack of *Rhizoctonia solani* and *Sclerotium rolfsii* (Lumpkin and Plucknett 1982). Since warm temperature coupled with high humidity favour insect development, pest problem is more serious in wet climate of tropics, while these pests were not found to be a big problem in the relatively dry climate of Africa (Van Hove 1989).

USE OF *AZOLLA* IN RICE

Origin and History of *Azolla* Use in Rice:

According to Lumpkin and Plucknett (1981, 1982), China and Vietnam are the only countries in the world known to have a long history of *Azolla* cultivation, and may have been united when cultivation originated. There are different legends about the genesis of *Azolla* cultivation which are popular among Chinese and Vietnamese people.

According to Chinese legend, a poor farmhand in Wenzhou district of Zhejiang province fell in love with a slave girl owned by the landlord. The landlord made the proposition that if the farmhand could increase the early rice yield from 1.5 to 2.5 t/ha, and late rice from 2.25 to 3 t/ha, he will allow him to marry the slave girl. The couple pondered how they could meet this seemingly unattainable demand. One night both the lovers saw a similar dream. They dreamed that while the boy was working in the field a celestial goddess appeared in the clouds with a melody and threw several flowers which made a bright light as they fell. He ran to catch them, but stumbled, causing the dream to end. The next day the couple started searching for the flowers and finally found some mysterious plants floating in a spring, and called them "Lu Ping" due to their colour, free-floating nature and shape (In Chinese Lu means green and Ping means free floating or wandering) and named the goddess as Ping Fairy goddess. They thought that these plants were sent to help them to fulfill their hope of being married. The boy placed some plants into one of the paddy field of the landlord, and the next day he was surprised to find that each plant had increased to nine plants. In two weeks the whole field was covered with *Azolla* and after 20 more days enough green manure for all the rice fields was produced. Due to *Azolla* the rice yield increased and their hope was fulfilled. In the gratitude for *Azolla*, the Wenzhou people constructed temples and shrines for the Ping Fairy goddess, which were dismantled during Cultural Revolution in the late 1960's (Lumpkin and Plucknett 1982).

According to one of the Vietnamese legends, a peasant woman named Ba Heng from the village of Lavan in Thai Binh province discovered and domesticated "Beo Giong" or "Beo Hou Dan" (floating mulberry flowers) *Azolla*. The *Azolla* was so effective as a green manure for the winter rice crop that a pagoda was erected in the honour of Ba Heng, and prayers and sacrifices were offered to her spirit (Lumpkin and Plucknett 1982).

In addition of above Vietnamese legend, Tuan and Thuyet (1979) reported that a Buddhist monk promoted the use of *Azolla* in Red River delta during 11th century and temples, which reportedly still exist, were built in his honour.

The use of *Azolla* in China is reported back to at least in the record of Ming Dynasty (1368-1644 AD) agricultural books (Lumpkin and Plucknett 1982, Watanabe 1994). The history of *Azolla* use in China indicates that its oldest use was in Wenzhou area. It was grown as intercrop in 200,000 ha in the south-eastern area in China during 1949 and by 1951 it was grown in 260,000 ha. During this period, the production, sale and distribution of *Azolla* was limited and the price of *Azolla* inoculum was quite high being US$ 13-20 or more for 100 kg of *Azolla* in early spring prior to field cultivation. In 1959 the Chinese government decided to investigate the potential of *Azolla*, and research for its nursery maintenance was improved during 1970's and by 1980 its cultivation expanded to about 13 provinces in China covering more than 1,300,000 ha (Lumpkin and Plucknett 1982). According to Liu (1979) *Azolla* was used in 1.34 million hectare in China.

Similarly in Vietnam, the farmers of La Van village were expert in multiplying *Azolla*, especially in summer; and used to sell it to other farmers, who used it as biofertilizer in their rice field (Nguyen-Cong-Tieu 1930). Its use was limited to about 40,000 ha at the time of independence and then government built an extension network of over 1,000 inoculum production bases to stimulate its utilization and it spread to about 700,000 ha in 1978 (Tuan and Thuyet 1979) and even more area in 4 provinces of Vietnam by 1980 (Lumpkin and Plucknett 1982).

The translation of Chinese literature into English in Hawaii and handing it over to International Rice Research Institute (IRRI), Philippines during 1977 helped in spreading the *Azolla* knowledge in other countries. The FAO organized tour on *Azolla* propagation and biogas technology to China in 1978, and symposium on Nitrogen and Rice at IRRI in 1978, also disseminated information on *Azolla* from China, and stimulated interest in this plant in other countries (Lumpkin and Plucknett 1982, Watanabe 1994). By 1980 in addition to China and Vietnam, it was used by researchers in Bangladesh, Burma, Egypt, India, Indonesia, Malaya, Nepal, Peru, the Philippines, Senegal, Sri Lanka, Thailand, USA (Hawaii and California), West African Rice Development Association (WARDA), and Office de Research Scientifique et Technique Outre Mer (ORSTOM) in Africa (Lumpkin and Plucknett 1982).

In 1979, by the joint cooperative project of Ministry of Agriculture and IRRI, *A. pinnata* was introduced in South Cotabato areas in Mindanao island of Philippines and during 1981-82 its use was spread to about 5000 ha. During 1982-83 *Azolla* growth slowed down in this area due to insect attack and long drought, then *A. microphylla* was introduced in 1982 and it gradually spread in this area (Watanabe 1982). The use of *Azolla* as biofertilizer was tested in 10 countries through International Network on Soil Fertility and Fertilizer Evaluation for Rice (INSFFER) trials organized by IRRI and results were summarized by Watanabe (1987). The value of *Azolla* as N fertilizer and its comparison with urea, using ^{15}N labelled *Azolla* and urea was demonstrated by the IAEA/FAO joint project (Watanabe 1994) during 1985-87 in 9 countries and the results of this joint project have been summarized by Kumarasinghe and Eskew (1993).

Suitability of *Azolla* to Rice Ecosystem:

Rice ecosystems, given below, are characterized by elevated rainfall pattern, depth of flooding and drainage, and by the adaptation of rice to these agroecological factors (IRRI 1994).

The *Upland* rice is direct seeded in non-flooded, well drained soils on level to steeply sloping fields. Crops suffer from lack of moisture and inadequate nutrition leading to very low yield. Upland rice is about 19 million ha worldwide and dominant in Africa, Latin America, but relatively less important in Asia.

The *Rainfed Lowland* rice is grown (transplanted or direct seeded) in puddled soils on level to slightly sloping, bunded or diked fields with variable depth and duration of flooding, being 37 million ha worldwide and dominant in humid and subhumid tropics.

The *Flood-Prone* (deep water) rice is seeded or transplanted in the rainy season on fields having medium to very deep flooding (50-300 cm), occupying 10 million ha, predominantly in South and Southeast Asia.

The *Irrigated* rice is grown in puddled leveled soil, bunded fields and with good water control. The crops is heavily fertilized and can reach 5 t/ha in wet season and more than 8 t in dry season. Irrigated rice is planted on 81 million hectare worldwide. It is dominant in Asian semiarid and subhumid regions, and accounts for 55% of worlds harvested area, and contributes to 76% of global rice production (IRRI 1994).

These figures show that in more than 87% (128 million ha) of the total area, rice is grown in flooded conditions which are also suitable for *Azolla* cultivation. The most popular and traditional legume crops are very good as nitrogen fixing green manures. But, inspite of their usefulness, the following constraints hamper their use by the farmers (Palaniappan 1992).

* In intensively cropped areas, farmers do not wish to set apart 6-8 weeks exclusively for growing a green manure having no direct cash benefit to them.
* After wheat harvest in April, the land may be fallow before rice, but the farmers find it difficult to do the farm operations in the intense heat of May and June.
* The high labour cost and high opportunity cost of land use for green manure. For example, the overall added cost to produce a green manure was estimated to about Rs. 500/ha which was equal to the price of about 90kg N in the form of urea. Thus, instead of growing the green manure, which is laboreous and time consuming, the farmers prefer to buy the chemical fertilizers which are easier for them and also show quick results on the crop.

In addition to above mentioned constraints for raising legume green manures, they have some weaknesses for rice farmers. Most of the rice soils are flooded during rice season and unfortunately most of the legumes cannot grow or fix nitrogen in such conditions (Lumpkin and Plucknett 1981). On the contrary to legume green manures, *Azolla* has no such weaknesses, rather these conditions are favourable for its growth and nitrogen fixation as this water fern is adapted to aquatic environment. The legumes need separate time and space for their cultivation whereas *Azolla* can be grown as intercrop with rice and thus requiring no separate land or water and time for its cultivation.

It is reported that *Azolla* can double its biomass in less than 2 days in laboratory conditions (Peters and Ito 1984), and in fields 3-5 days in favourable conditions, while in 5-10 days in normal conditions (Joshy 1983, Khan 1983, Lumpkin and Plucknett 1980, Watanabe et al 1977), indicating a much higher growth rate than most of the legumes.

Thus, the adaptation of *Azolla* to aquatic habitat, very high rate of biomass production and very high rate of nitrogen fixation, makes it the most suitable biofertilizer for rice ecosystem.

Laboratory Cultivation and Transportation:

Germplasm collection is necessary to maintain the genetic variability of an organism and to screen and select the desirable type for direct use or for genetic enhancement. In the Soil Microbiology Department of International Rice Research Institute (IRRI) P.O.Box-933 Manila, Philippines, a total of 529 accessions by Aug. 1993 are preserved (Watanabe 1994), and the description of IRRI collection of 501 strains by 1992 is given by Watanabe et al (1992). They have reported that this collection, originating from 55 countries, comprised of 56% accessions from Asia and Oceania, 4.2% from Europe, 24.8% from North and Latin America and 7.8% from Africa. According to them *Azolla* strains are being preserved in vegetative stage because all the strains do not sporulate and progeny of sporocarps also show genetic segregation, and they are being transferred to shoot-tip cultures as shoot-tip culture can be maintained longer (2-3 months) than vegetative cultures (14 days). In addition to IRRI the Azolla accessions are duplicated in Laboratory Physiologie Vegetale, Catholique Universite de Louvaine, Belgium and at National *Azolla* Research Centre at Fuzhou by the Fujian Academy of Agricultural Science, China (Watanabe 1994).

The importance of laboratory cultures of *Azolla* cannot be denied as all the studies like morphology, cytology, ontogeny, pathology, physiology, ecology and genetics are dependent largely on successful laboratory cultivation of the organism. In addition to above studies, laboratory cultures are useful to maintain the culture in pure form for culture collection.

Azolla species collected from natural environment can usually be easily cultured in the laboratory and well maintained green house conditions. The field samples collected for laboratory cultivation are routinely surface sterilized with a solution containing 0.12% sodium hypochlorite and 0.01% Triton X-100 as a wetting agent, washed with large volumes of sterilized water and transported to sterilized nutrient media with and without combined N source (Peters and Calvert 1982).

In case of shoot tip culture, after washing the healthy fronds, 5mm fragments including meristem and several leaves are cut-off from the old parts. After sterilization for they are washed with sterile water and 15-20 pieces of the cut tip portions are inoculated into N-free IRRI *Azolla* medium having 0.5% agar, however for *Anabaena*-free *Azolla* 2 mM NH_4NO_3 is also added into culture medium (Watanabe et al 1992).

A variety of inorganic nutrient solutions have been used for *Azolla* culture including N-free modification and dilution of; Crones, Knopes and Watanabe (IRRI) media (Peter and Ito 1984), ASM-of Gorham et al (Ashton 1974), Hoagland solution (Costa et al 1994, Watanabe et al 1992) and specially designed media for some physiological studies (Kitoh and Shiomi 1991, Yatzawa et al 1980). Becking (1979) has summarized the composition of 11 culture media comparing cation and anion concentration as well as initial pH already mentioned under Ecophysiology.

In modified N-free IRRI medium of Watanabe et al (1977) buffered at pH 6 with 10 mM MES (2N-Morpholinoethane sulfonic acid) at optimum temperature for different species (25 and 30°C) and at photon flux density of 400 and 600 $\mu E/m^2/s$, Peters and Ito (1982) got doubling of *Azolla* in 1.4-2.0 days having 5-6% N on dry weight basis. However Peters and Calvert (1982) did not consider the inclusion of the above said buffer essential in the culture medium.

To maintain *Azolla*, stock cultures can be conveniently kept for several months between transfers on nutrient solution solidified with 2% agar, in gauze-cotton caped Fernbach or Erlenmeyer flasks (Peters and Calvert 1982). The beaker culture is also suggested for *Azolla* maintenance. In this technique 150 ml of N-free IRRI medium in 250 ml beaker coated with black paint from the 150 ml mark to bottom, to prevent algal growth, is used. The *Azolla* inoculated beakers are incubated at 26/18°C day/night temperature, 15 K lux light intensity for 12 hours/day, and relative humidity of 70-75% (Watanabe et al 1992). They have also given the details of better cultivation system which is automatic and more useful for maintaining a large culture collection. It comprises of 200L capacity tank having Hoagland culture solution modified by substituting KCl and $CaCl_2$ for KNO_3 and $Ca(NO_3)_2$, and diluted by adding 2 parts of the medium to 3 parts of water. Different strains of *Azolla* contained in small individual nets (strainer) and placed in a small room having 25/18°C temperature, 55-70% humidity, light of 16 Klux (265 $\mu E/m^2/s$) at 10 hr/day from

a bank of neon tubes and tungsten bulbs. The nutrient solution level is automatically controlled with a float and its circulation is carried out with a pump from main tank to *Azolla* culture tank.

For transportation or shipment from one place to other, *Azolla* has to be taken in the vegetative form as most of the strains do not produce spores. Since *Azolla* may be contaminated with some insects or other unwanted guests, insecticide and fungicide treatment is necessary. Such treatments are particularly essential for samples collected from fields. The contaminated plant material is also not allowed to enter into some developed countries like USA (Lumpkin 1987). Because *Azolla* is sensitive to desiccation, samples should be transported as fresh material in moist conditions in a container or plastic bag. The excess water should also be drained out to save it from rotting and turbulence leading to excessive fragmentation and often death of plants (Lumpkin 1987, Watanabe et al 1992). Regardless of the duration of shipment, *Azolla* should not be exposed to temperatures below 0°C or above 35°C (Lumpkin 1987). During long-distance travel, *Azolla* should be kept in hand-carried baggage and stored in a refrigerator during stay at hotel or in air-plane and this way it can survive for at least 2 weeks. When samples are sent by air mail, preferably by courier services, *Azolla* may survive for 10 or more days depending on the transportation conditions (Watanabe et al 1992).

If fertile *Azolla* having micro-and megasporocarps is found then whole plants bearing sporocarps can be air dried (in the shade) and this dried material, which can survive for months, can be shipped to distant places. After arrival, the dried material can be placed in pure water, in indirect sunlight to induce germination of *Azolla* spores. After a few days the container should be lightly agitated to cause the male and female spores to make contact and then within 2-3 weeks small seedlings should be present and can be moved to a suitable nutrient solution (Lumpkin 1987).

Nursery Cultivation:

The cultivation of *Azolla* is a continuous process through the year, as at present its propagation through vegetative material is known in detail. Secondly, most of the strains do not sporulate and the process of spore induction and large-scale harvesting is not known and

germination is insufficiently understand (Lumpkin 1987b, Watanabe 1994). Therefore, special outdoor nurseries are prepared to maintain *Azolla* throughout the year.

The maintenance nurseries are usually small in size and number, and require very intensive and careful management. These may need special structure for temperature maintenance, special water supply and nutrition measures and intensive pest and disease control and are located near place of residence or farm house where management problems are minimum and regular supervision by the caretaking person is possible (Lumpkin and Plucknett 1982, Van Hove 1989).

Over-Wintering:

In cold areas, like high latitude sub-tropical or temprate zone it is often necessary to protect *Azolla* from damage due to low freezing temperature. For safe over-wintering shelter from cold wind, ice and snow is considered essential and prevention from freezing is a main concern. Areas with hot springs or warm water from some factories (which can pass through the nursery for warming) are especially suitable. Where warm mater is not available, *Azolla* can be grown in shallow water (3-10 cm deep) in glass or plastic houses. In China, 0.5-0.6m high arch-shaped plastic green houses, made with arch bamboo strips were used commonly. In these green houses *Azolla* nurseries measuring 10 X 1m having 13-20 cm depth were made and positioned in east-west direction, so that the whole bed is exposed to south to receive maximum winter sunlight. During sunny days about half of the plastic was rolled back to allow direct exposure to sun on south side and apply fertilize to *Azolla* nursery. In less cold areas a smaller roll-away mat and cone shaped move-away straw mats are also used. Wet-soil cultivation is also useful as soil temperature is usually higher than water and nutrient stress is also reduced. *Azolla* loosely piled in a stack, covered with straw-ash, and routinely sprinkled with water to keep from drying is also practiced in China (Lumpkin and Plucknett 1982, Lumpkin 1987). In area where winter is not so severe and freezing temperatures are only occasionally experienced *Azolla* can be grown in a part of lake or pond having nutrient-rich water, as due to larger body of water a more stable water temperature is maintained. In a large lake in northern Chekiang area in China during a continuous one-week snowfall in February, the lowest water temperature was still 11°C and during winter it was stable between 13-15°C. (ZAAS 1975).

For winter maintenance nurseries, about 0.35 kg/m^2 fresh *Azolla* plant density (heavy stage) is desirable because *Azolla* survives best when the mat is heavy but not too crowded. When *Azolla* mat begins to become wrinkled and plants begin to display different colours the mat is thinned to heavy stage again.

Fertilizer during cold weather differs from other seasons and in addition to phosphorus some nitrogen and potash are also required. A basal application of 750 g FYM/m^2 along with 15 g superphosphate/m^2 is used, and during growth period a top dressing of spray solution containing 10 kg calcium phosphate dissolved in 50 kg cattle urine and diluting the mixture to 300 L of water is also applied (Lumpkin and Plucknett 1982).

As *Azolla* species vary for cold tolerance, cold tolerant varieties of *A. filiculoids* and *A. rubra* which can survive at freezing temperature are more useful (Lumpkin and Plucknett 1982, Watanabe et al 1992).

Over-Summering:

Over-summering techniques are used in locations where field temperatures are too high for *Azolla* cultivation or where *Azolla* must be preserved through a hot season for later use. The best sites for these nurseries have good ventilation, direct sunlight and cool flowing water (Lumpkin 1987). Sufficient light is also essential as long-term cultivation in excessive shading resulted into disease, infection and death of *Azolla* in China (ZAAS 1975). However in tropical countries where water temperature exceeds 40°C for long periods, wooden frames about 1 m high, having reeds or straw mats on top, are used to shade the *Azolla* nursery during daylight hours (Lumpkin and Plucknett 1982). *Azolla* can also be over-summered between shading bands of rice plants or in the shade of tall herbaceous plants such as *Sesbania* that are planted along the edge of rivers, ditches and ponds. Sometimes, *Alternanthera philoxeroides*, *Eichornia crassipes* and *Pistia stratiotes* are intercropped with *Azolla* to provide shade (Lumpkin 1987). In addition to shading, moist or shallow water cultures were tried in hot summer (Liu 1987).

A nutrient rich water, with a steady level is desirable, as *Azolla* growing in water having a low level of nutrients turns reddish, has small fronds, long roots, and poor biomass production in hot days; as compared to green and healthy plants in nutrient rich water. Phosphorus application helps to tolerate heat to maximum extent and nitrogen fertilizer,

especially at higher concentration, is harmful. When *Azolla* has been damaged by heat putrefied barnyard fluid should be applied alongwith phosphate fertilizer or 1% ammonium sulphate solution should be sprayed. After this treatment, as soon as the *Azolla* plants turn greenish or has a red central part with green outermost leaves, the nitrogen application should be stopped as higher amounts of ammonium sulphate for longer periods of application kill *Azolla*. For improving *Azolla* growth during summer, about 9t well rotten barnyard (farmyard) manure/ha should be applied as basal fertilizer, and 1.5-2% superphosphate solution should be sprayed as top dressing (during cooler hours) after every 5-7 days (ZAAS 1975).

In Egypt, *Azolla* was grown in small nurseries measuring 2x4mx0.2 m (depth) having 4-6 cm water, in groups of 4 or more, made with cemented lining. Soil-water culture amended with chicken manure @ 1 kg/nursery (1.25 t/ha) or low amount of superphosphate (30 kg P_2O_5/ha) was used for maintaining *Azolla* nursery. The tapping with broom was also done to enhance vegetative multiplication (El-Bassel et al 1994).

For growth of *Azolla* in Sri Lanka, outdoor tanks of 2.8X1.85 m having a 3 cm layer of washed sand, and flooded to 5 cm level above the sand surface with tap water, were used. It was found that application of poultry manure to supply 1g P/m^2 resulted into about 3 times *Azolla* biomass as compared to same amount of P as superphosphate (Kulasooriya et al 1994).

Overcrowding (>0.5 kg fwt/m^2) reduces heat tolerance by *Azolla*, and encourages pest or fungal attack of *Rymanae* or *Rhizoctonia* especially in humid weather, therefore, frequent thinning and dispersal of *Azolla* mat and flushing with clear cool water is required for successful cultivation. When diseased *Azolla* is noticed it should be removed as soon as possible and remaining one should be sprayed with 0.1% solution of ethyl-mercuric chloride or 0.3% solution of dinitrophenyl thiocyanate. For algal control 1% solution of $CaCO_3$ may be helpful (Lumpkin and Plucknett 1982).

Multiplication Nurseries:

The purpose of these nurseries is to produce *Azolla* in sufficient quantities to inoculate rice fields at the proper time. A few weeks before *Azolla* inoculation is needed on a large scale, multiplication of *Azolla* is carried out in rice field in multiplication nurseries

(Lumpkin 1987b). For such nurseries, leveled fields having good soils of medium fertility, pH 5-7, and with 5-7 cm water depth, are suitable. The field should be divided into smaller plots to reduce wind and wave action. Depending upon the amount of inoculum available from maintenance nursery, the multiplication nursery should be inoculated at the rate of 0.2-0.4 kg fwt/m^2. In low P soils, superphosphate should be applied at the rate of 5-10 kg P_2O_5/ha/week to enhance *Azolla* growth (Lumpkin and Plucknett 1982). To increase vegetative multiplication, *Azolla* mat should be patted with an *Azolla* beater, which looks like a broom and is made from about 10 small, thin bamboo branches, arranged in a fan like fashion with a 5-7 feet long flexible bamboo handle (ZAAS 1975). As soon as *Azolla* covers a plot of the multiplication nursery, the ridge (bund) of the adjacent plot is removed to double the area for *Azolla* multiplication (Watanabe 1982).

Field Cultivation:

When *Azolla* inoculum is needed in large scale for rice fields, it is often necessary to use large fields for multiplication nurseries. There are three different systems used for cultivation of *Azolla* as a (i) monocrop and incorporated into paddy mud as a basal green manure before transplanting rice, as an (ii) intercrop with rice and either incorporated as a top dressing green manure or allowed to grow as cover to die naturally without incorporation or (iii) both a monocrop and an intercrop (Lumpkin 1987b, Roger et al 1993).

Monocrop Cultivation: In China and Vietnam when water is available and fields are fallow then *Azolla* is grown for 20-30 days before rice and is incorporated into paddy mud once or twice during this period by ploughing or harrowing (Lumpkin and Plucknett 1982, Lumpkin 1987). Since *Azolla* is incorporated a few days before rice transplanting, its decomposition brings a good supply of nitrogen, with phosphorus and potash as well, to the growing rice during the early period of its growth (Van Hove 1989).

For monocrop cultivation of *Azolla*, paddy fields are irrigated, usually without ploughing the soil to reduce labour cost. The presence of stubble of previous crop prevents the accumulation of *Azolla* to one side due to wind. However if the soil is ploughed before *Azolla* inoculation, the uneven ploughed surface have the same effect as the stubble in the first case. The water level is usually maintained at less than 5 cm (2.5-5 cm) depth, for keeping the *Azolla* close to soil for better nutrient uptake.

Azolla has population density of less than 1 kg (fresh wt.)/m^2 during exponential phase and 1 to 4 kg/m^2 during linear phase and it should have a density of 2-4 kg/m^2 at incorporation if possible. The very low inoculum rate favours the development of weeds and very high rate reduces its growth rate. The recommended inoculum densities vary from less than 0.1 to 1 kg/m^2, however inoculum between 250 and 500 g/m^2 generally represent a good compromise and should only be exceeded in exceptional cases. To get sufficient *Azolla* in shorter period, multiple nurseries close to or midst of rice fields should be arranged (Van Hove 1989).

A low level of inoculum i.e. 25 g/m2 or more may be used if the flooded fields are going to stand for one month or more. While for very short fallow period much higher levels (300 g/m2) will be required. Inoculum rate usually in China ranges from 3.5-5 metric tons/ha (350-500 g/m^2) for monocrop cultivation (Lumpkin and Plucknett 1982).

The inoculation of *Azolla* may be done by broadcasting, or along with water flowing from a field at upper level to the lower level which is possible in hilly and other fields having different levels. During broadcasting of inoculum it is usually in the form of clumps that are formed after harvesting and transportation. The clumps reduce growth therefore plants should be dispersed by striking with the *Azolla* beater (broom).

Depending on the amount of available inoculum and amount of *Azolla* to be needed, different periods of growth will be required. For example assuming a doubling time of 7 days and to obtain a 50t *Azolla* mat/ha: 53, 46, 39, 32, 25 and 18 days will be required for the inoculum rate of 0.25, 0.5, 1, 2, 4 and 8 t/ha, respectively (Lumpkin and Plucknett 1982).

In Vietnam, the half-saturation method is recommended for *Azolla* cultivation and saturation density of *A. pinnata* is about 10-20 t fresh weight/ha. In this method available inoculum is spread in an area to make density of 0.5 kg fwt/m^2. After one week, when surface is fully covered, the area for this *Azolla* is doubled. Again, by the end of 2nd week when *Azolla* covers this area, the present *Azolla* is spread into double of this present area. Thus by repeating this procedure, the area covered by *Azolla* is exponentially expanded. For example inoculum of 10-15 kg fresh *Azolla* in an area of 20 m^2, will become 40 m^2 after 1 week (having 20-30 kg *Azolla*) then it will become 80 m^2 after 2nd week (having 40-60 kg

Azolla), and will expand to 160 m^2 (having 80-120 kg *Azolla*)after 3rd week, and then to 320 m^2 (having 160-240 kg *Azolla*)after 4th week, of initial inoculum (Watanabe 1982).

To obtain adequate growth of *Azolla*, weekly or biweekly application of fertilizers are usually required. The growth rate and *Azolla*-N content are reported to increase when available (Olsen P) is more than 25 ppm and P adsorbing capacity is less than 4.4g/kg of soil (Roger and Watanabe 1986). However, in most of the paddy soils, P is the most common element limiting *Azolla* growth (Ali and Watanabe 1986, 1987, Watanabe 1994). The P deficient *Azolla* plant signals by turning its leaves red or reddish-purple, then soluble P like superphosphate should be applied at the rate of 0.5-1.0 kg P/ha/day, in the form of powder and sometimes mixing with fine dry FYM, or as a liquid spray as 1-2% solution which is more useful than dry forms (Lumpkin and Plucknett 1982). Split application is better than basal dose and 1 kg P_2O_5/ha every 4 days is recommended and Vietnamese recommend 5-10kg superphosphate (1-2 kg P_2O_5/ha) every 5 days (Watanabe 1982) while 4-5 kg P_2O_5/ha/week was recommended in Indian rice soils (Singh 1979). In suitable conditions each kg of P_2O_5 results into 2 kg additional N in *Azolla* biomass (Watanabe 1982).

In severely depleted or light (Sandy) soils potassium is often used at the rate of 2-5 kg/ha and in Vietnam wood or kitchen ashes are applied at a rate of 100 kg/ha (Lumpkin and Plucknett 1981, 1982). In Vietnam, the use of 10 kg K_2O or 100 kg ash/ha/10 days along with P was recommended by Tuan and Thuyet (1979).

Since at higher pH iron availability is reduced (Brady 1984), therefore, for *Azolla* growth at pH 8-8.5 in California, iron as FeEDTA was applied at the rate of 0.8 kg Fe/ha and it enhanced *Azolla* growth (Talley et al 1977). Application of 1.0 kg Fe/ha has also been reported by Singh (1992) to increase growth and nitrogen fixation in *Azolla*.

Availability of Zn is more in acidic pH and critical point is near 7 above which its availability declines, due to its precipitation as $Zn(OH)_2$ and zincate ion with Ca, and under flooded conditions due to formation of sulphide (Brady 1984). According to De Datta (1981) next to N and P, Zn deficiency now ranks first among the nutritional disorders that limit rice yield and broadcasting about 5-10 kg zinc sulphate/ha (and more) after puddling was useful for increasing rice yield in calcareous soils. In Thailand, at one site, even the daily application of P did not lead to healthy *Azolla* growth and Fe chelate had to be applied, and incidentally significant response to Zn was observed (Swatdee et al 1979).

The application of 1.5 ppm Mo as sodium molybdate after 4 days of *Azolla* inoculation led to more *Azolla* growth in Egypt where soil pH decreased from 8.2 to 7.0 after flooding due to organic matter decomposition (Yanni et al 1994). The use of 0.15 kg Mo/ha was reported to improve growth and nitrogen fixation (Singh 1992) while application of about 0.3 kg of molybdate/ha at small intervals was considered sufficient by Lumpkin and Plucknett (1982).

Both the Chinese and Vietnamese use organic manures as basal fertilizer for *Azolla* cultivation. Decomposed animal manure, night soil, ashes and compost are all used to improve *Azolla* growth as they supply all the essential nutrients required for *Azolla* growth. (Lumpkin and Plucknett 1982). In China the use of barnyard manure at the rate of 10 catties/mou (about 100 kg/ha) as basal fertilizer and the application of 2 catties of superphosphate as top dressing improved *Azolla* growth, however FYM was better than chemical fertilizer, especially for revitalization of weak *Azolla* plants (ZAAS 1975). The application of 500-1000 kg FYM/ha after every 5-10 day was also recommended for increasing *Azolla* growth (Roger and Watanabe 1986).

Pest damage is a serious problem, particularly in hot summer ($>30^{\circ}C$) because generation time of insects decreases at higher temperature (Watanabe 1982) and the different pests (already mentioned under ecophysiology) like larvae of lepidopterous insects and different types of water snails damage *Azolla* mat seriously. Application of 3-4 kg actove omgredoemt a.i. carbofuran (Furadan) or ferithion per hectare is effective for pest control, particularly along with phosphorus fertilizer. The dipping of *Azolla* inoculum in 1000-3000 times diluted insecticide solution is also effective for pest control (Watanabe 1982). In India, the use of natural pesticides like *Neem* oil, *Karanj* oil or *Neem* cake have been reported not only to control the pests but also to stimulate *Azolla* growth, while for fungal control, 0.5-1.5% Benlate solution was recommended (Singh 1992).

As the *Azolla* mat grows thicker, it is often necessary to disperse the mat so that individual plants can grow and spread further. For this purpose *Azolla* beater is used to pat the surface of the mat gently but not beating vigorously, to speed up the coverage of water surface by *Azolla* (Lumpkin and Plucknett 1982, ZAAS 1975)

When the *Azolla* mat becomes too dense, its growth will decrease and mat will begin to display wrinkles on its surface. This is the proper time to incorporate it into soil. To

facilitate incorporation of *Azolla* into soil, irrigation may be stopped or field is drained off of excess water. *Azolla* may be incorporated into soil manually by hands (FAO 1979) or by animal pulled or engine driven weeders (Lumpkin and Plucknett 1982).

A tight but even mat of *A. pinnata* was reported to have a biomass of 10-20 t/ha in warm weather or 20-40 t/ha in cool weather and even up to 100 t/ha for *A. filiculoides* (Lumpkin and Plucknett 1982).

If a second crop of *Azolla* is to be raised after first incorporation, then the monocropped *Azolla* is incorporated immediately after draining the water (without drying the mud) with scattered puddling so that a part of the mat remains unincorporated for next multiplication for monocrop growth or for culturing as intercrop with rice to be transplanted in the same field.

Intercrop Cultivation: When water or land is not available, or environmental or cultural limitations do not allow for growing *Azolla* as monocrop, then it may be grown concurrently with rice in the inter-row areas in the same field using no extra space or water for its growth. As an intercrop, *Azolla* can be inoculated into the field just before or after transplanting the rice, however inoculation before transplants is more common as its broadcasting and spreading is easier than in the presence of transplanted rice seedlings. Depending upon the availability of *Azolla*, it may be inoculated at the rate of 0.3-0.4 kg/m^2 for one day ahead or on the same day as rice is transplanted, or 0.5-0.6 kg/m^2 for 5-7 days after rice transplanting. However, if insufficient *Azolla* is available 0.02-0.05 kg/m^2 may be inoculated and it can cover the field in 20-40 days after inoculation (Lumpkin and Plucknett 1982).

According to Roger and Watanabe (1986), intercropped *Azolla* is usually not fertilized but if superphosphate is available one application of 4.5 kg P/ha per crop is recommended. To reduce the early inhibition effect (explained below), application of chemical fertilizer-N is recommended (Roger et al 1993). Secondly, the application of N before rice planting will promote tillering in rice as *Azolla*-N is available at later stage after its decomposition (Singh and Singh 1990). The other management practices like pest control are similar to monocrop cultivation.

The intercrop system of *Azolla* with rice leads to some new factors due to their mutual cultivation, some effects are negative but transitory, while others are of more long

term nature (Lumpkin and Plucknett 1982, Van Hove 1989, ZAAS 1975). First *Azolla* competes with rice for nutrients, however, this period of competition lasts for about 10-20 days after *Azolla* is inoculated usually until the first incorporation of *Azolla*, and in China this phenomenon was referred to as early inhibition of rice. However, this early inhibition ends after nitrogen and other nutrients from the first incorporation of *Azolla* become available to rice. Then, after 2nd incorporation, more nitrogen is available to rice and positive effects are more prominent and lead to more panicles per hill and Chinese call this "late promotion" of rice by intercropped *Azolla* (Lumpkin and Plucknett 1982, ZAAS 1975).

The incorporation of *Azolla* may be done manually by hands (FAO 1979), which is a perfect method but laborious, or with small rotary rice weeder (conventionally used by farmers or new version developed by IRRI) for which the rice must be planted in rows (Lumpkin 1987b). To avoid the labour of incorporation, Chinese and Vietnamese also use a process and call it "natural lodging". In warm and humid weather, usually at maximum tillering stage of rice, when *Azolla* mat becomes too dense and thick it is allowed to be attacked by fungi, which occurs naturally in such situation. Due to fungal attack the *Azolla* mat dies and decays to release nitrogen and other nutrients, which are utilized by growing rice. Unfortunately, much of the nitrogen may be lost through run-off and denitrification before it can be absorbed by the rice, but the practice is accepted as a compromise because of low labour demand of "natural lodging" versus the high labour required for hand incorporation, and was the traditional method used in Vietnam and China (Lumpkin and Plucknett 1982).

NITROGEN FIXATION

The high growth rate and nitrogen fixation capacity of *Azolla* has attracted researchers for its use as nitrogen biofertilizer in rice based cropping system (Khan 1983, Lumpkin 1987b, Lumpkin and Plucknett 1982, Singh 1990, 1992, Watanabe 1982, 1994). The nitrogen fixation by *Azolla* is estimated to be comparable to legumes and much more than free living bacteria and blue-green algae (Stevenson 1982, Watanabe 1986). According to Roger and Ladha (1990) the range of nitrogen fixed by the rice rhizospheric bacteria is 1-40 kg N/ha/crop under optimum conditions, by blue-green algae 0-80 kg N/ha/crop, by *Azolla* 20-224 kg N/ha/crop and by legume green manures 20-212 kg N/ha/crop.

Azolla growth rate is very high and under optimum light and temperature conditions Peters and Ito (1984) has obtained doubling time of about 2 days or even less for *A. filiculoides, A. caroliniana, A. mexicana* and *A. pinnata*, which corresponds to about 0.34g/g/day. Ashton (1974) has reported growth rate of around 0.2 g/g/day during exponential phase and it increased with increase in day length but decreased in severe crowded conditions.

In liquid culture medium doubling time of about 2-5 days was observed for *A. filiculoides* by Talley and Rains (1980) and also for *A. pinnata* by Watanabe et al (1977). In India, increase in fresh biomass of 2-6 fold every week for *A. pinnata* was reported (Singh 1979, Singh and Singh 1990). Even when certain environmental conditions are not optimal, as in field conditions, the doubling time may be around ten or fewer days (Roger et al 1993).

Although *Azolla* may absorb nitrogen from its aquatic environment, its endophytic nitrogen fixing blue-green algae *Anabaena azollae* is capable of meeting the entire nitrogen requirement of this association even at doubling time of less than two days (Peters and Ito 1984). The direct evidence of nitrogen fixation by *Azolla-Anabaena* symbiosis is its growth in nitrogen free medium and by $^{15}N_2$ uptake, while indirectly by reduction of acetylene to ethylene by the nitrogenase activity of its *Anabaena*. During nitrogen fixation N_2 is reduced to NH_3 and ^{15}N studies have revealed that isolated symbiont released upto 60% fixed nitrogen as NH_3 into medium (Lumpkin and Plucknett 1980, 1982, Peters et al 1980).

The nitrogen fixation in *Azolla* varies with environmental conditions, therefore different researchers have reported different figures. Moore (1969) concluded that *Azolla* may assimilate 100-160 kg N/ha in 3-4 months corresponding to 1.2 kg N/ha/day. In USA, Talley et al (1977) observed nitrogen accumulation of 41 kg and 52 kg N/ha in 35 days for *A. mexicana* and *A. filiculoides* corresponding to 1.2 and 1.5 kg N/ha/day respectively. In IRRI fields, continuous growth of *A. pinnata* for one year gave an average rate of 1.4 kg N/ha/day (Watanabe 1978). The nitrogen fixation was found to be variable between 1.0 to 2.6 kg N/ha/day, being upto 2.8 for *A. filiculoides* and 3.1 kg N/ha/day for *A. pinnata* in open paddy fields in Philippines (Watanabe 1982). Roger and Watanabe (1986) have reported 0.4-4.6 kg N fixed/ha/day, with an average of 2 kg after evaluating the data from different sources, and FAO (1988) also estimated 2-4 or more kg N/ha/day. Under ideal conditions,

it has a potential of fixing more than 10 kg N/ha/day (Singh 1992), while Lumpkin and his Chinese co-workers found as high as 10.5 kg N/ha/day in a cooperative experiment in China (Lumpkin and Plucknett 1982). When *Azolla* is grown as dual crop with rice the average nitrogen fixation by *Azolla* may be 0.4 to 2.9 kg N/ha/day (Tuan and Thuyet 1979).

In open field conditions a layer of *Azolla* formed in 7-20 days after its inoculation, contain about 10t green manure per hectare providing about 20-30 kg N/ha as it contains about 4-6% dry matter and 4-5% N on dry weight basis or 0.2-0.3% on fresh weight basis. This amount of nitrogen by *Azolla* can be doubled and tripled by growing two or three or more layers of it after incorporation of first crop (layer) during a rice crop season, so reaching a value of 450 kg, or even more than 800 kg N in a year (Kikuchi et al 1984, Singh 1979). it was reported that in China 10 harvests of *Azolla* in 100 days produced 157.5 ton green material containing 428 kg N, corresponding to 931.3 kg urea or 2142 kg ammonium sulphate (FAO 1979). Even considering the lowest rate of nitrogen fixation of 0.4 kg N/ha/day it can fix 25 kg N/ha/60 days, in dual culture of rice and *Azolla*. However under favourable conditions, 130-170 kg N fixation is possible per hectare in a two month period (Tuan and Thuyet 1979).

It has been estimated that rice requires about 20 kg N/ton of harvested crop and a normal rice crop of 6 t/ha of grains removes an average of 120 kg N (Lumpkin and Plucknett 1982). Thus it is possible to supply more nitrogen than required by a normal crop of rice, and Liu (1979) has shown the possibility of obtaining 12-15 tons of rice yield per hectare, depending only on the nitrogen supplied by *Azolla* grown in the same field.

Unlike other free-living or symbiotic nitrogen fixers, *Azolla-Anabaena* shows nitrogenase activity and continues to fix nitrogen in the presence of significant quantities of combined nitrogen (Becking 1976, Kitoh and Shiomi 1991, Peters et al 1981, Talley et al 1977, Watanabe et al 1977) because the nitrogen fixing alga resides in the aerial lobe, which is not submerged and thus not in direct contact with the nitrogen of the medium (Becking 1976, Talley et al 1977). This property of *Azolla* makes its use compatible with the chemical fertilizer use of present day agriculture system.

Decomposition and Availability of *Azolla*-N to Rice:

Field and laboratory data suggested that ammonical nitrogen was released directly into water by *A. mexicana* cover (Rains and Talley 1979, Talley and Rains 1980). Shen et al cited by Lumpkin and Plucknett (1980) has reported secretion of nitrogenous compounds into aquatic environment and release of 14-21% fixed nitrogen into water from a Chinese *Azolla* variety called Whole River Red. They have also quoted Brill, reporting that in his laboratory about 20% of fixed N_2 was excreted as ammonia by a specimen of *A. mexicana*. About 5mM ammonium accumulation was observed in the culture medium and herbicides enhanced this process (Zimmerman 1992). Due to the ability of nitrogen fixation and to pump out ammonia, it is hoped that *Azolla* can be used as a living biosolar-driven system for ammonia production in future (Hamdi 1982).

Although some ammonical (fixed) nitrogen is released directly into the water. The nitrogen fixed by *Azolla-Anabaena* symbiosis is mainly available only after its decomposition and mineralization (Moore 1969, Watanabe et al 1981). The non-incorporated *Azolla* also decomposes after dense mat formation and over crowding stimulates its decomposition (Singh and Singh 1990). However its decomposition is reported to be faster when incorporated into soil than left as cover (Singh 1992, Talley et al 1977, Watanabe et al 1981). *Azolla* biomass, when incorporated into soil, decomposes and its nitrogen along with other nutrients is gradually released during different stages of rice growth (Alejar 1982).

The herbaceous *Azolla* plant has less woody tissues and has a narrow C:N ratio of 7:1 to 18:1, facilitating a faster decomposition of its biomass (Lumpkin and Plucknett 1982). In laboratory studies Tuzimura et al (1957) found decomposition and mineral N release from soil containing *Azolla*, and it was as high as released from green soybean addition. They also observed that two third mineral nitrogen (NH_4+NO_3) was released in 5-8 weeks in aerobic conditions at 29°C. Watanabe et al (1977) has also reported about 75% of total N mineralization in 6-8 weeks of incubation. Li (1984) has reported that the release of NH_3 reached a peak (20% mineralization) after 2-3 weeks of incorporation into soil and which declined at 9th week. Alejar (1982) observed about 12% NH_3-N released by 2nd week and maximum of upto 21% during 12th week in light, while 21% mineralization occurred during 10th week in dark conditions, indicating rapid mineralization in later case. Watanabe et al (1981) reported that at 30°C, mineralization for different species of *Azolla* was rapid during

first week of incubation and it slowed down after 3rd week, and it was noted that the lower the N content of *Azolla* strain the less mineralized its nitrogen was. Singh (1979) reported that mineralization of *Azolla* incorporated into soil released about 56% N as ammonia in 3 weeks when incubated at 24°C, whereas 80% was released at room temperature. Singh and Singh (1990) reported release of 41-67% of its N within 7-35 days in India.

The mineralization of ^{15}N labelled *Azolla* was about two times faster in mineral soil having 2.4% organic matter(OM), C/N 14 and pH 4.7, as compared to organic soil having 21.8% OM, C/N 19 and pH 4.1. It was observed that about 30% of added N was in the KCl-N form by 2nd week, 50% by the 4th week and 60% by the 16th week of incubation in case of mineral soil, while only 27% of total N was mineralized by 16th week in organic soil, and no losses of nitrogen were observed during incubation period of *Azolla* in these soils. In case of mineral soil, incubation of *Azolla* for 16 weeks, there was approximately 60% KCl-N, 10% amino-N, 10% hexosamine-N, 10% in unknown form and 10% as insoluble-N (Rabeharisoa 1994). The laboratory incubation of *Azolla* as indicated by the changes in labelled exchangeable N in soil showed that at least 65% of *Azolla*-N was mineralized within 10 days (Watanabe et al 1989). Ramirez (1983) studied decomposition of fresh *Azolla* by using different species in flooded soil and on the average she found 14, 29, 31, 35, 37, 40, 44 and 44% mineralization during Ist, 2nd, 3rd, 4th, 5th, 6th, 8th and 10th week of incubation, respectively. This N release pattern from *Azolla* indicated that the gradual release of *Azolla*-N can efficiently be used by rice plant during different stages of its growth and such release will also reduce N losses from the rice soil.

The fresh *Azolla* is reported to decompose faster than dried *Azolla* and 75% of its N is released in 6-8 weeks (Singh and Singh 1990) and release of N from *Azolla* compost is reported to be much slower (Singh 1989). As compared with chemical fertilizers, Singh and Singh (1990) has reported that release of N from chemical fertilizer was about 87% within 10 days whereas release of *Azolla* N was comparatively slower.

As regards the availabilityof *Azolla*-N to rice, it has been reported that most of *Azolla* tissue decomposes after 8-10 days of its incorporation in Indian soils and rice plants are benefited which is noticeable after 20-30 days (Singh and Singh 1990). The increase in nitrogen recovery from *Azolla* to rice was higher for *Azolla* incorporated into soil than allowed to decompose in water (Talley et al 1977). The studies using ^{15}N labelled *Azolla*

indicated that its N availability to rice in a pot experiment was 53 and 10% for *Azolla* incorporated and water-surface-floated *Azolla* respectively, however in field conditions these values were 26 and 13% respectively (Watanabe et al 1981). As compared to chemical N fertilizer, *Azolla* N release was slower and its availability to first crop of rice was about 70% of that of ammonium sulphate (Watanabe et al 1977). According to You et al (1987) the incorporation of ^{15}N labelled *Azolla* and urea at panicle initiation of rice indicated 13 and 10% nitrogen derived from *Azolla* and fertilizer into rice respectively, while ^{15}N recovery was 56 and 61 mg/pot for *Azolla* and urea respectively. Watanabe et al (1989) have reported the ^{15}N recovery of *Azolla*in the first crop of rice to be 39% from basal application and 63% from the side dressing. In a IAEA coordinated trial in 9 different countries, it was found that generally the ^{15}N recovery by rice plant was not very different from ^{15}N labelled *Azolla* or urea, when applied in the same manner whether at transplanting or at maximum tillering, and ^{15}N recovery was mostly higher from application at maximum tillering than from application at transplanting. The ^{15}N recovery from different *Azolla* species was different due to quality of the material (C:N ratio, fiber content etc) and ^{15}N recovery in rice from fresh, frozen and dried *Azolla* was 33, 23 and 21% respectively (Kumarasinghe and Eskew 1993).

Effect of *Azolla* on Rice Yield:

Positive effect of *Azolla* has been reported on the agronomic characters of rice plant including quality of rice grain and paddy yield by different workers (Moore 1969, Lumpkin and Plucknett 1982, Watanabe 1982, Singh 1992, ZAAS 1975). It was found that number of shoots (tillers), length of longest leaf, fresh weight and dry weight of rice was higher due to *Azolla* green manuring (Lumpkin and Plucknett 1980). It was observed that plant height and number of tilers in rice increased due to *Azolla* (Singh 1979, 1989) and number of fertile tillers, number of grains per panicle, and panicle weight was significantly higher if *Azolla* was incorporated at early stages of rice growth period (Singh and Singh 1987).

The number of productive tillers were increased due to *Azolla* incorporation in Egypt (Yanni et al 1994). In Sri Lanka, 32% more filled grain per panicle than control were observed by Kulasooriya and de Silva (1977). Rice grains obtained from *Azolla* inoculated plots were better formed and were denser, than treated with manure alone (Nguyen-Cang-Tieu 1930).

and much higher protein content in rice was found due to *Azolla* than when equivalent N fertilizer was used (Lumpkin and Plucknett 1982).

Although *Azolla* improves rice plant and rice grain quality yet it also increases the rice yield being the major interest of researchers and rice farmers. Nguyen-Con-Tieu (1930) reported from Vietnam that rice yield was 1764, 2500 and 3500 kg/ha in control, manure (1800 kg/ha) and plots receiving manure (900 kg/ha) plus *Azolla*, respectively. From China it was reported that in a commune the rice yields were 3-3.6 t/ha before *Azolla* use, but in 1977 due to *Azolla* use at the rate of 21 t/ha the paddy yield rose to 9 t/ha, being about 3 times increase in rice yield (FAO, 1979).

In Nepal an increase of 17% in rice yield was observed due to *Azolla* (Joshy, 1983). The summary of 1,500 experiments in China indicated that fields manured with *Azolla* gave higher yield by 600-750 kg/ha, and statistics from 422 field experiments in a province indicated increase in yield in 99% cases and the average was 18.6% (Liu 1979). The trials at IRRI, Philippines in dry season, for *Azolla* incorporated at 40 days after transplanting caused 12-25% (0.3-0.7 t/ha) increase of paddy yield over control, while inoculation of *Azolla* before as well as after rice transplanting produced a maximum of 36% (1.9 t/ha) increase in rice yield over control (Watanabe 1978). In the Philippines (South Cotabato) upto 32% (1.12 t/ha) increase in rice yield was found for Azolla over control (Kikuchi et al 1984). As compared to control the increase in rice yield in Thailand was 13-75% for *Azolla* when grown and incorporated before and with rice, over control (Lumpkin and Plucknett 1982), and it ranged from 30-170% increase in other trials (Swatdee et al 1979). In *Azolla*-rice experiments in Taiwan the increase in rice yield was 40-46% for two *Azolla* species (Lumpkin and Plucknett 1982). In Sri Lanka in a plot experiment the incorporation of *Azolla* containing 30 kg N/ha resulted into about 50% increase in rice yield (Kulasooriya et al 1994). In Bangladesh increase in rice yield ranged from 5 to 50% due to *Azolla* application (Miah and Amin 1991). In India, Singh (1979) observed 52% and 39% increase in rice yield for *Azolla* incorporation and allowing it to decompose without incorporation respectively. To see the impact of *Azolla* and fertilizer on rice yield in Model Agronomy trials at 4 locations in India it was found that there was a 33-95% increase in yield due to *Azolla* (Singh 1992). In Egypt, the inoculation of *A. caroliniana* led to about 50% increase in rice straw as well as in grain yield (Yanni et al 1994). In United States, Talley et al (1977) in

small plot studies found 22, 65, 113 and 207% increase in rice yield over control for *A. filiculoides* cover, *A. mexicana* cover, *A. filiculoides* once incorporated, and *A. filiculoides* once incorporated plus cover, respectively. Rains and Talley (1979) reported that dual culture of *A. filiculoides* caused 25% increase, while incorporation of two crops of *A. mexicana* resulted into three times increase in yield as compared to unfertilized control. In *Azolla* trials carried out in 10 countries at 37 sites under the International Network on Soil Fertility and Fertilizer Evaluation for Rice (INSFFER) programme of IRRI, about 20% higher rice yield was obtained for un-incorporated (cover) *Azolla* and 35% for *Azolla* incorporated treatment (Watanabe 1987). The *Azolla* incorporation trials at the rate of 60 kg N/ha in different countries, showed a 25-140% increase in rice grain yield over control (Kumarasinghe and Eskew 1993).

In some of the *Azolla* experiments, organic manure or chemical fertilizers were also used to compare and compute the benefits of *Azolla* equivalent to chemical fertilizer-N. In Vietnam Nguyen-Cong-Tieu (1930) observed that due to *Azolla* plus 900 kg FYM/ha, the rice yield increase was more than using only manure at 1800 kg/ha, and thus due to *Azolla* more than 900 kg FYM/ha can be saved. From China, Su (1983) reported that the effect of *Azolla* incorporation before transplanting of rice at 40 t/ha fresh weight was equivalent to 60 kg N from ammonium sulphate while an additional 20 t/ha of *Azolla* incorporation at mid-tillering stage was equivalent to 78 kg N from ammonium sulphate. At IRRI, Philippines growing rice in wide rows (53 cm) alternating with narrows rows (13 cm) and hills 6.6 cm apart (Watnabe (1982) showed that 4-6 of *Azolla* incorporations gave rice yield equivalent to 70-100 kg N/ha from chemical nitrogen fertilizer application. In Nepal the increase in rice yield due to single incorporation of *Azolla* was equivalent to 30 kg N/ha (Joshy 1983). In India, incorporation of fresh *Azolla* at the rate of 8-10 t/ha was as effective as 30-40 kg N/ha of ammonium sulphate, while incorporation of 10-12 t/ha of *A. pinnata* along with 30 and 50 kg N/ha as ammonium sulphate gave yield that was comparable to that obtained with 60 and 80 kg of this chemical fertilizer (Singh 1977 cited by Singh 1990). The average of three seasons showed that one basal incorporation and twice during intercropping gave rice straw and grain yield equivalent to application of urea at 60 kg N/ha (Singh 1990). In 15 years studies at CRRI, India it was found that *Azolla* provided 20-40 kg N as basal green manure while 20-30 kg N ha as dual cropping with rice and value of upto 90 kg N/ha may be achieved for

one basal plus twice incorporation during dual cropping (Singh 1992). In Bangladesh, increase in rice yield due to *Azolla* was equivalent to 40 kg N/ha (Miah and Amin 1991). In USA, Talley and Rains (1980) found that incorporation of *A. filiculoides* at 40 kg N/ha produced two tons of extra grain yield which was comparable to yield obtained by 40 kg N/ha from ammonium sulphate. In summarizing the results of INSFFER trials Watanabe (1987) has reported that in 7 different countries at 33 locations, the incorporation of one crop of *Azolla* before or after rice transplanting increased rice yield equivalent to that obtained from 30 kg N/ha as urea or ammonium sulphate during 1979-1980; while in 1981-1982, 32 trials in 8 countries showed that incorporation of two crops of *Azolla* was equivalent to 60 kg N/ha of urea.

Effect of *Azolla* on Floodwater and Soil Properties:

Floodwater: A thick *Azolla* mat does not allow sunlight to penetrate the water, so the water temperature remains cooler under *Azolla* during the day time, and a temperature difference of 3°C was observed in China during a clear day (ZAAS 1975). In addition to effect on temperature, *Azolla* can reduce oxygen level and increase CO_2 level in the soil of rice fields, and it was observed that soil redox potential droped markedly due to its respiration and organic matter build up with *Azolla* maturity (Lumpkin and Plucknett 1982, ZAAS 1975). Because of lower water temperature, and slower wind action the *Azolla* cover is also reported to reduce water losses cause by evaporation (Diara and Van Hove 1984).

In China it was found that pH of paddy water rose to 9.5 on neutral soil in control whereas it was around 8.6 for *Azolla*-covered water and the pH increase was more prominent between 10 am to 3 pm. The increase in pH was attributed to algal growth which consumed CO_2 from floodwater in non-*Azolla* water, as application of $CuSO_4$ to kill algae or shading resulted into almost no algal growth in water and thereby no much increase in floodwater-pH was observed (Tuan and Thuyet 1979).

Weeds: The maxim that "Any plant is a weed when it grows where it is not wanted" is seldom appropriate for *Azolla* as it is a desirable plant in rice fields (Lumpkin and Plucknett 1980). Weeds compete with rice for nutrients and water, leading to decrease in its yield, and about 22% reduction in rice yield was reported in Pakistan (Saleem 1994). The fertilizer as weed population and

thereby almost doubls the labour requirement for hand weeding to obtain maximum rice yield (O'Brien & Price 1983). Weed suppression effect of *Azolla* has been reported from China (Liu 1979, ZAAS 1975), United States (Talley et al 1977, Rain and Talley 1979). In India weed biomass in the presence of *Azolla*, especially if inoculated in higher amounts, was reduced to 65% of uninoculated control (Kannaiyan 1987), while 70 and 93% reduction in *Echinochloa crusgalli* was reported for 50% and 100% *Azolla* cover respectively (Ngo cited by Lumpkin and Plucknett 1980). Agricultural economists have estimated that Asian farmers spend more of their time in weeding than other activities related to rice production, and the benefit of *Azolla* due to weed suppression may be sometimes more than as nitrogen source (Lumpkin and Plucknett 1981).

From Vietnam, Nguyen-Cong-Tieu (1930) reported that under *Azolla* cover weeds such as *Urticularia flesnosa, Panicum crusgalli* and *Sagittaria* species get very little sunlight and eventually die off, and the weeding expenses can be reduced, thus a saving of $4/ha was possible. Kikuchi et al (1984), for *Azolla*-rice field experiment in Philippines, computed the saving of weeding labour of $7/ha due to *Azolla* mat.

Azolla forms a light-proof mat on water surface so light starvation of young weeds occurs and this mat also offers physical resistance to weed growth. But the rice seedlings are not affected, as they stand above *Azolla* mat when transplanted (Lumpkin and Plucknett 1981).

Soil: *Azolla* when used as green manure and incorporated into soil improves the physical properties and fertility of the soil due to its organic matter and release of nitrogen and other nutrients (Lumpkin and Plucknett 1982, ZAAS 1975, Zhang 1987, Watanabe and Ventura 1992). The field studies in China showed that soil bulk density decreased from 1.28 to 1.15 g/cm3 while pore space increased from 3.7 to 4.2% and granular structure was improved by 50% due to *Azolla* and the benefit in soil improvement was equivalent to application of 15-20 t/ha of pig manure (ZAAS 1975). The improvement in soil physical properties helps in easy tillage and thus reduces energy input and this loose soil also allows profuse development of crop roots (Liu 1979, Lumpkin and Plucknett 1981, Shin et al 1978). Increase in cation exchange capacity (CEC) from 13 to 27 meq/100g soil due to *Azolla* was reported from Indonesia (FAO 1988).

Azolla absorbs nutrients from water which might be washed away in run off. The analysis of 126 samples of 7 species of *Azolla* showed that P content ranged 0.2-1.6%, Ca 0.5-1.7%, Mg and also S 0.2-0.7%, Si 0.2-3.5%, Na 0.2-1.3%, K 0.3-6.0%, Fe 0.04-0.59% Mn 66-2944 ppm, Cu 0-264 and Zn 26-989 ppm, on dry weight basis (Lumpkin and Plucknett 1982). Thus the release of these nutrients after its decomposition helps in cycling of nutrients and these nutrients along with biologically fixed nitrogen are available to rice plant (Lumpkin and Plucknett 1981).

Shin et al (1978) reported that after *Azolla* has been grown for a period of time, the soil turns black and becomes "alive", and *Azolla* incorporation was better than other materials for soil organic matter improvement as application of 50 kg (dwt) of *Azolla*, *Astralgus* and rice straw led to 39, 26 and 31 kg of organic matter in soil respectively. Liu (1979) reported from China that after cultivation of *Azolla* in the field, the soil became darker in colour and had 0.09% more organic matter than check. Similarly, 0.05% increase in humus was reported due to *Azolla* incorporation in China (FAO 1979) and increase in organic matter from 0.05 to 0.16% was reported for *Azolla* cultivation in rice fields (ZAAS 1975). Increase in soil organic matter and water-soluble N was reported to be 10 and 11% respectively due to *Azolla* use (Wang and Wang 1987). In India, increase in organic carbon, N, P, K and availability of micronutrient like Fe and Mn in soil as a result of *Azolla* incorporation was reported (Singh 1992). The availability of S and Zn to rice was higher from *Azolla* than control (Mian et al 1991), and the availability of N, P, K from fertilizer was enhanced upto 52, 46 and 72% respectively, when fertilizers were applied with *Azolla* (Zhang 1987).

The use of *Azolla* as green manure improves soil organic matter thereby soil properties like granular structure, bulk density, pore spaces, aeration, drainage, and infilteration and nutrient supply increases and all these properties are essential for soil fertility, required for obtaining high crop yields and sustainable agriculture.

Residual Effect of *Azolla* on Subsequent Crop:

The long term studies of *Azolla* decomposition showed that after 5 years about 36-39% of added *Azolla*-carbon remained in soil under water logged conditions and 21-22% under upland conditions (Wen et al 1987). Using ^{15}N labelled *Azolla* Watanabe et al (1981)

found that availability of *Azolla*-N to first rice crop was 53% when it was incorporated and only 10% when it was floated on the water surface, while in field conditions these values were 26% and 13% respectively. These studies and other work mentioned previously under 'nitrogen fixation, decomposition and N availability to rice, show that all of the *Azolla*-N maynot be utilized by the first rice crop. It is also said that when amount of *Azolla* applied to rice has more nitrogen than required by rice or when all of *Azolla*-N is not utilized by rice, the left over *Azolla*-N may lead to higher yield of crops following rice, due to its residual effect (Singh 1992).

The researchers at Hangzhou, China, have reported that there was some residual effect of *Azolla* green manure in the second rice crop, which disappeared in the third crop (FAO 1985). In India, the incorporation of *A. pinnata* at the rate of 1t/ha along with fertilizer at 10 DAT of rice, not only increased rice yield but a higher response (due to *Azolla* application during rice) was obtained in the following Rabi crop, and was partially attributed to residual effect of *Azolla* (Jayaraman 1990, cited by FAO, 1991). In Sri Lanka, a higher rice grain yield was obtained for the second rice crop in wet season and the higher yield in second crop, which was more prominent for *Azolla*+urea treatment, was attributed partially to residual effect of *Azolla* (Kulasooriya et al 1994). The long time use of *Azolla* as green manure in rice fields in Thailand indicated that during first two years chemical fertilizers gave higher rice yield than *Azolla*. However during 3rd, 4th and 5 year, the use of *Azolla* before or after rice transplanting, gave higher rice yield than chemical fertilizer; and improvement in soil fertility, particularly in low-fertility soils, was also reported (Loudhapasitiporn and Kanareugsa 1987). At IRRI, the second crop of rice was about 54% higher in *Azolla* applied soil than control, and the other crops i.e. 3rd to 5th were also higher for *Azolla* than control, and the higher yield was attributed to carry-over of nitrogen from *Azolla* from the previous crops (Watanabe et al 1981). The ^{15}N labelled *Azolla* studies in different countries indicated that at the end of the first rice crop 11-64% N of *Azolla* remained in soil, and 2-12% was recovered in the succeeding rice crop, however, the yield of subsequent rice crop for *Azolla* treated plots (during Ist crop) was almost equal to control in Thailand, but was more than double in Indonesia (Kumarasinghe and Eskew 1993). In China about 20-30% higher rice yield of 2nd crop was obtained for *Azolla* than control, while the yield of winter wheat, (the 3rd crop) was about double in plots having

Azolla+fertilizerthan fertilizer only, indicating that residual effect of organic + inorganic fertilizer was more than chemical fertilizer and may extend to 3rd crop also (ZAAS 1975). A long term study at IRRI, indicated that the 10th crop of rice was 0.5t/ha higher in both the *Azolla* as well as *Sesbania* treated plots than control and urea treated plots. Similarly the N uptake in the 10th rice crop was 11 kg N/ha higher for *Azolla* than urea and control plots and this amount of N accounted for 10% of the N added in the previous crops. The almost equal grain yield and N uptake in the urea and control plots, indicated that there was no residual effect of urea, as observed for *Azolla* or *Sesbania* after nine consective rice crops (Watanabe and Ventura, 1992).

OTHER USES

Use as Green Manure and Compost:

Although *Azolla* is grown as green manure primarily for rice, it can also be used as green manure or for compost formation, for upland crops. Records going back to 540 B.C. indicate the use of *Azolla* as animal feedstuff in China (FAO 1979). Thus, in addition to its use as biofertilizer, it can also be used as feed for fish, poultry and livestock, for energy in biogas plants, and as decontaminant of polluted water (Lumpkin and Plucknett 1980, 1982, Van Hove 1989, Watanabe 1991).

Azolla has been reported to be grown as biofertilizer with some plants growing in aquatic habitat, like water bamboo, arrowhead (*Sagittaria* sp.) and taro (*Colcasia* sp.) in China (ZAAS 1975).

Azolla as fresh, dried or in the form of compost can be used as organic fertilizer for other crops than rice, and also for horticultural and ornamental plants (Lumpkin and Plucknett 1982, Khan 1983). In some parts of China in addition to its use as green manure, its compost is added as fertilizer to wheat, maize and rape at the rate of 30 t/ha; and fertilizer trials in different provinces showed that increase in yield due to *Azolla* was over 800 kg for wheat, 710 kg for beans and 945 kg/ha for maize (FAO 1979). Positive effect of *Azolla* manure on corn yield has also been reported from Mexico by Ferrera-Cerato and Romero (1982).

For making compost different plant materials having different compositions are used. The C:N ratio of most straw material is very wide being as high as 100:1, narrow in

legumes (and farm manure) being 20-30 (Brady 1984), but it is narrower in *Azolla* being generally 12:1 (Lumpkin and Plucknett 1982). So the quality of rice-straw compost can be improved by mixing it with *Azolla*, and the resulting *Azolla*-rice straw compost will be of better fertilizer value containing C 26, N 2, P 0.23, K 1.2 and Fe 0.44% while Mn 1033, Copper 10 and Zinc 35 ppm (Khan 1983).

Use as Feed and Food:

Azolla is used as fish food and it was found that fish like grass carp and *Tilapia* showed a marked preference for *Azolla* and *Lemna* than other aquatic plants (Lumpkin and Plucknett 1980, Antoine et al 1987). As *Azolla* contain about 22-37% crude protein on dry weight basis and also contains the 3 essential amino acids namely: lycine, methionine and histidine (Khan 1983), it can be a good fodder for cattle, swine, and poultry particularly ducks and chicken and forage for fish (Khan 1983, Lumpkin & Plucknett 1980, Van Hove 1989).

In the feeding tests by *Tilapia nilotica* in Japan, the results showed that about 20% of fish feed can be replaced with *Azolla* and thus making the feed more economical (Shiomi and Kitoh 1994).

The use of *Azolla* as fish fodder was considered to be more practical and useful in China. In the rice-*Azolla*-fish system, the excreta of fish increase the growth of *Azolla* and phytoplanktons, and the fish consume both these plant materials as fodder, and rice field provides place for multiplication and growth of both *Azolla* and fish (Liu 1988, Van Hove 1989). The growth of rice, fish and *Azolla* in the same time and space, increases the economic value of *Azolla* in this system (Watanabe 1991).

Because of high food value, *Azolla* is also suggested for human consumption (Lumpkin and Plucknett 1982, Khan 1983, Watanabe 1991). Some appealing recipes based on *Azolla* namely, soups, *Azolla*-plus-meatballs have been mentioned, and for this purpose *Azolla* should be grown in satisfactory hygienic conditions (Van Hove 1989). Dr. Manzoor Khan (author of 'Primer on *Azolla*' 1983) has personally told me that he had eaten *Azolla* in Philippine served by some farmer, and it tasted like spinach and there was no digestive problem.

Use for Biogas and Water Purification:

The anaerobic fermentation of *Azolla* after mixing it with rice straw in a biogas digester results in production of methane which can be used for lighting or heating, and the effluent from the digester can be used as fertilizer for the neighbouring agricultural crops (FAO 1979, Van Hove 1989).

Azolla has been reported to help in purification of waste water (Lumpkin and Plucknett 1980, Watanabe 1991). In Japan, *Azolla* was tested to remove nutrients from sewage. *Azolla* was grown in multistory tray system using a continuous flow of waste water, and a model of waste water treatment by using fish and aquatic plant like *Azolla* was suggested to purify the domestic sewage and agriculture waste water (Shiomi and Kitoh 1987).

CONSTRAINTS, ECONOMICS AND POTENTIAL FOR FUTURE

Constraints and Possible Solution:

Despite the long history of *Azolla* use in China and Vietnam and success in Philippines, its use is limited due to environmental and social constraints (Lumpkin and Plucknett 1982, Lumpkin 1987, Singh 1992, Watanabe 1991,1994).

Water: The primary constraint for use of *Azolla* is its requirement for an aquatic habitat. Some *Azolla* must be maintained all the year round and multiplied in nurseries prior to large-scale field cultivation, thus a certain amount of water must be available throughout the year for maintenance of *Azolla* nursery. For inoculum preparation, about 20% of the total area to be seeded, is required for *Azolla* nursery, which should have a regular supply of good quality water; and for cultivation in the fields, some water at least at some places, must remain for its multiplication during rice growth period (Lumpkin 1987).

To economize water use, the same amount of water can be applied in smaller amounts with lesser depth at regular intervals, instead of a few heavy irrigations. It will not only help in maintaining *Azolla* growth at a constant rate, but will also reduce leaching losses of water and nutrients, away from the root zone. The use of moist-soil culture is also recommended for water economy in *Azolla* cultivation and more than 50% to around 90% *Azolla* yield was obtained for different species for moist cultivation at two sites in China (Liu

1987). But the use of moist-culture may not give yield equal to water culture at all locations and the rate of multiplication may be slow (Lumpkin 1987).

The cultivation of *Azolla* through spores is considered to be the ideal method and it eliminates the cultivation of nursery throughout the year and also reduces transportation problem. The spores are said to be more tolerant to adverse conditions than vegetative parts, however growth from sporocarp germination to full growth of sporophyte plant, ready for inoculation into field requires 40-60 days, and sporulation is possible only in limited number of *Azolla* species e.g. *A. filiculoides* in China, *A. microphylla* and *A. mexicana* in Philippines (Watanabe 1994).

The spore-culture method to seed *Azolla* sporocarp on soil-bed was developed by Chinese in early 1980s (Lu 1987), but due to lack of spore formation in most of the species and maintenance of optimum water and temperature condition during spore germination, the technique has not spread to field level.

Temperature: After water, temperature is probably the most important environmental factor limiting the spread of *Azolla* cultivation, and is also a factor most difficult to control by man, and this problem is considered to be more serious in the tropics (Lumpkin and Plucknett 1982). Presently, *Azolla* is maintained in the vegetative form in the nursery and the year round maintenance of *Azolla* culture requires not only sufficient water but a reasonable temperature for its growth. Thus high temperature close to 40°C as experienced in Pakistan or India and low temperature below 10°C as found in some temperate regions or in hilly areas, may kill *Azolla* and become a problem for its cultivation in open fields (Beri and Meelu 1983, Watanabe 1994).

The over-wintering and over-summering techniques (mentioned under *Azolla* cultivation in Review section) can help in maintenance of *Azolla* in the nursery. However, in field multiplication heat tolerant strains of *A. mexicana*, *A. microphylla*, *A. caroliniana* and *A. pinnata* may be useful (Lumpkin 1987).

The introduction of foreign *Azolla* may be more successful than the indigenous one, as in Philippines *A. microphylla* replaced the indigenous *A. pinnata* in one province, while *A. caroliniana* and *A. mexicana* were widely spread in other places. Similarly *A. filiculoides* from Germany grew very well in China (Watanabe 1994). In Senegal, *A. pinnata* introduced probably from India, spread in early 1980s, while *A. microphylla* and *A. pinnata* var.

imbricata and other introduced *Azolla* species into W. Africa adapted well in semi-arid climate and grew as good as or even better than that which was found in their native places in Asia (Nguyen 1994).

Phosphorus: Of the inorganic nutrients essential for *Azolla* growth, phosphorus is considered to be the most limiting one under field conditions (Ali and Watanabe 1986, Watanabe 1994). The survey of Philippines showed that at least half of the *Azolla* sample taken from rice fields, canals, and ponds were deficient in P (<3.3 g/kg dwt, in *A. pinnata*), and 20-25 mg of available P/kg soil was considered to be the critical level for P deficiency. Similarly P deficiency in *Azolla* was observed in most of the samples collected from Thailand (Watanabe 1994).

For alleviating P deficiency problem for *Azolla* growth, P loaded *Azolla* grown with a smaller amount of P application into a smaller area of nursery will lead to higher concentration of P in the plant and this P loaded *Azolla* continue its multiplication for several times in the field conditions. Secondly, instead of application of large amounts of P at one time, split application in smaller doses (2-5 kg/ha) was considered more economical and efficient (Watanabe et al 1988, Watanabe 1994). Field cultivation of *Azolla* usually requires P application, therefore the amount of P intended for direct application to rice can be applied to *Azolla* for increasing its growth and N_2-fixation, and the same P will become available to rice, after the *Azolla* is incorporated into soil (Lumpkin 1987). The moist-soil culture helps in uptake of P directly from soil due to the penetration of roots into mud and thus P deficiency is releaved (Liu 1987). The use of low-cost P sources like animal manure has also been suggested for improving *Azolla* growth (Kulasooriya et al 1994).

The ability of different *Azolla* species to grow under P deficient conditions indicates that species like *A. pinnata* may be used in low P soils (Watanabe 1994).

Pests: The major biotic factor limiting the wide spread use of *Azolla*, particularly in humid tropics, is its susceptibility to pests and pathogens (Lumpkin and Plucknett 1982, Mochida et al. 1987). The high temperature, near 30°C and slightly higher one, alongwith high humidity accelerate pest growth. Thus the high population of pests damage *Azolla* plants and decrease its multiplication rate. For control of these pests, expensive pesticides are required in *Azolla* nursery, and particularly at large scale in the field conditions, which reduces the

economical benefit of *Azolla* for using it as an alternate source of fertilizer (Lumpkin and Plucknett 1982, Kikuchi et al. 1984, Watanabe 1994).

To overcome the pest and pathogen problems and reduce the input of pesticides different methods are suggested. According to Watanabe (1994) the insecticides should be applied to inoculum or *Azolla* maintenance nursery, while the use of economical and natural insecticides like Neem-cake, which not only reduce pest attack but increase nitrogenase activity, has been proposed by (Kannaiyan 1987). The use of fish for predation of *Azolla* pests, Rice-*Azolla*-fish culture has also been recommended (Liu 1988).

Since overcrowding or malnutrition often trigger pest and pathogen attack, *Azolla* should be incorporated before overcrowding and required fertilizer like P,K,Zn,Fe etc. should be applied, to minimize the damage (Watanabe 1994). The use of moist-soil culture, instead of flooding, helps to reduce the pest attack by restricting the larval movement of pests and also helps in absorbing nutrients directly from soil (Liu 1987), but this is applicable only to well drained fields (Watanabe 1994).

Since the resistance to pests varies with strain or species of *Azolla*, relatively more pest-resistant species of *A. pinnata* may be used in some localities (Mochida et al 1987).

Social and Economical: Biofertilizers, being slow-acting, do not readily manifest the beneficial effect of their use as mineral fertilizers do. Therefore, the farmers have to be convinced first about the benefits of biofertilizers through a process of their use over a period of time. Most of the work done on biofertilizers is mostly confined to research stations, government farms and demonstration plots, and organized extensive work remains to be done for creating better awareness among farmers on the benefits of biofertilizers. The use of *Azolla* may be intensified in areas where temperature, soil and flooded condition are favourable for its multiplication before transplanting rice or during rice growth (Beri & Meelu, 1983).

In addition to water and need of equipment/tools for *Azolla* incorporation the poor extension services in many countries of West Africa limit use of *Azolla* by rice farmers. Therefore, the committed efforts from the governmental and non-governmental organizations and support from international organizations was thought necessary for wide spread use of *Azolla* (Nguyen 1994).

Economic analysis of *Azolla* technology in the areas where *Azolla* was grown easily showed that it was economical for increasing rice yield (Kikuchi et al 1984). The incorporation of *Azolla* into soil is considered a laborious work. The use of animal or tractor driven rotary weeder, for incorporation of *Azolla* before rice transplanting is practicable (Lumpkin and Plucknett 1982). The use of automobile *Azolla* harvester for collecting and distribution of *Azolla* for mechanized *Azolla* cultivation was suggested to reduce the labour cost (Talley and Rains 1982).

Since many developing countries have neither nitrogen fertilizer producing industries nor the foreign exchange to import them and the expensive fertilizers remain inaccessible to small farmers, the use of *Azolla* as biofertilizer should be encouraged for crop production (Van Hove 1989).

On the whole, green manuring, a 3000-year old practice, which contributes all essential nutrients including nitrogen and have long-term economic advantages including sustainability factor, is very relevant in today's context, and must be made use of where feasible and economically viable in relation to total farming system operation (Palaniappan 1992). Secondly, while comparing the cost and yield benefit of *Azolla* with chemical-N fertilizers, its longterm benefit for concomitant increase in soil fertility for sustainable agriculture should also be considered (Roger et al 1993).

Economics and Potential for Future:

Although intensive research on *Azolla* has started recently, its practical use as source of organic N fertilizer for rice production has been recognized in Asia, particularly in China and Vietnam for quite a long time. This suggests that the use of *Azolla* in rice farming has been economically viable in these places. However, economic feasibility of the transfer of this technology to new areas, especially the tropical countries may depend on the local conditions (Kikuchi et al 1984, Rosegrant and Roumasset 1988). Since *Azolla* biofertilizer technology is labour intensive, it is economically feasible only in those countries which are manpower resourceful (Singh 1992). Considering the input costs of *Azolla* use like its application in the field and other non-land costs, and saving in N fertilizer input herbicide application, reduction in weeding labour cost and fertilizer application labour cost due to

ply of N (19 kg/ha) and

for maximum supply of N (50 kg N/ha) from *Azolla* respectively. It was further estimated that if *Azolla* could be adopted for rice production in all irrigated rice fields in Philippines, the total economic return could range from $17.6 to $64.2 million annually, being equal to or greater than the annual government fertilizer subsidy (Kikuchi et al 1984). They pointed out that if expensive insecticides have to be applied for controlling (only the) pest of *Azolla* then the benefits of *Azolla* use are not attractive. However, they concluded that economic potential of *Azolla* is great and its use is economical where environmental conditions are favourable for its growth and labour costs are also low.

In India, the cost of dual cropping of *Azolla* using recommended inoculum of 500 kg/ha was estimated to be Rs. 50-54/ha and cost of P fertilizer and pesticides was not included as they are also used for rice crop and P applied to *Azolla* is released into soil after its decomposition. But if the cost of labour, superphosphate and pesticide to grow 10 t *Azolla* (containing 20-30 kg N) are also taken into account then it comes to about Rs. 130 (Rs. 26 = 1 US$), which is equal to the price of 20 kg N of fertilizer. However the cost of *Azolla* can be reduced by using low cost sources of P such as animal dung and cheaper biopesticides. Thus, if the first estimate is considered then the use of *Azolla* is more attractive and it was estimated that investment of one rupee in *Azolla* dual cropping gave a net return of Rs. 21-24, as against a net return of Rs.5 with chemical N fertilizer, and so the use of *Azolla* was reported to be feasible in India which is a manpower resourceful country (Singh 1992). The use of *Azolla* as intercrop with rice was also computed to be more economical than its use as monocrop (Rosegrant and Roumasset 1988).

It is also pointed out that apart from supplying N to rice, long-term benefits of using *Azolla* such as improvement in soil fertility and increase in availability of other nutrients should also be given due weightage while comparing it with chemical N fertilizer (Roger et al 1993, Singh 1992).

The use of *Azolla* has proved to be beneficial in China, Vietnam and South America and it has a promising future in some regions, and better laboratory as well as field work strategies are needed to improve the effective use of *Azolla* (Van Hove and Lejeune 1994). The future use of *Azolla* depends on the germplasm pool and exploitation of its potential and on the effectiveness of research and development efforts (Lumpkin and Plucknett, 1982). According to Watanabe (1986), a common characteristic of technologies currently adapted

by farmers for *Azolla* use are labour intensive and require different inputs. It is unlikely that nitrogen biofertilizers could be an exclusive N source for producing high yield in economically feasible conditions. So most probably the future of biofertilizer technology in rice cultivation is its integration with other technologies, including proper use of N fertilizers i.e., N fertilizers that will give the least interference to nitrogen biofertilizers and basic research is needed to develop these technologies to solve field problems.

If, with the integrated research, the present problems of *Azolla* are solved jointly by the efforts of biologists to improve *Azolla* plant capable of growing in low P water, fixing more amount of nitrogen, propagation through spores, resistant to pests; and by agronomist to find low-cost P fertilizers and optimize the use of inputs; and by engineers to develop implements for its incorporation; then its use becomes more economical (Lumpkin and Plucknett 1982). The development of *Azolla* strains, through research, having minimum constraints are also considered essential for its economical use (Rosegrant and Roumasset, 1988).

Rice accounts for 60% of the 500 million tons of cereals harvested annually in developing countries in Asia. If 10% of the rice area in developing countries in Asia being about 5.2 million hectare could produce an extra 1t rice/ha, due to incorporation of 10t/ha of *Azolla*, the additional 5.2 million tons of rice produced will be sufficient to feed nearly 15 million people annually, thus investment on *Azolla* research could be easily justified (Lumpkin and Plucknett 1982).

The achievement of specifically desired agronomic attributes like high temperature and salinity tolerance, high nitrogen fixation and ammonium leaky *Azolla* (Sandhu 1982, Aleem 1985), induction of spores (Burris 1994) are more likely to be acquired through controlled intraspecific and perhaps interspecific crosses of the host rather than by changes in the symbiont population (Peters 1991). According to Watanabe (1994) improved *Azolla* hybrids have been obtained in China and further genetic improvement of *Azolla* for desirable characters is possible through sexual hybridization and algal exchange, and the multiple use of *Azolla* like green manure, compost and feed for animals will improve its economic benefits in future.

MATERIALS AND METHODS

AZOLLA SURVEY IN RICE AREA

Soil, Climate and Cropping System:

In Pakistan rice is grown in about 2.097 million hectare, being 1232, 692, 110 and 63 thousand hectare in Punjab, Sindh, Balochistan and North Western Frontier Province (NWFP) respectively, and more than half (54%) of the total rice area is in Punjab (MINFA 1992). According to Chaudhary (1978), the cultivation of rice is concentrated in to four distinct zones (Fig. 3), and the most important land qualities common to all these areas are abundant water supply during summer, relatively low position in landscape, restricted drainage and slow permeability of the soils. All the Pakistani rice soils are developed in calcareous alluvium of mixed mineralogy and illite clay is dominant while montmorillonite is also found but rarely.

As mentioned above that rice in Pakistan is restricted to 4 major zones and more than half of this area is situated in the northern area of Punjab called Northeast Indus plans zone 2 (Fig.3). This zone is a broad strip of land in the lower apron of the Himalayan piedmont, especially between Ravi and Chenab rivers and comprises extensively clayey basins and channel infills. The soils in this area are nearly flat, relatively high-lying deep, well drained, silty clay-loam, calcareous alluvium of mixed mineralogy classified as typic Ustochrepts or Eutric cambisols. The soils of Gujranwala series have been leached free of lime to about 1m depth, underlain by a thick lime - accumulated horizon with abundant lime nodules. The pH ranges from 7.5 to 8.0 and organic matter content about 0.6-0.8% and cation exchange capacity is about 20 meq/100g. The plough layer, about 10-15 cm thick, like in all rice soil is mottled with brownish and grayish concentration of iron and manganese oxide in a grayish matrix (Chaudhry, 1978). According to Hasan et al (1993) the soils of rice area are loam to clay loam, having pH 8.0-8.4, EC 0.84-3.1 dS/m, Olsen's P 3-20 mg P/kg, organic matter 0.51-1.03% and ammonium acetate extractable-K 132-351 mg K/kg.

The soil in this zone are under a subhumid, subtropical climate with about 400-700 mm rainfall in the monsoon period of July to mid-September and depression area are subjected to seasonal flooding. The zone has a typical continental climate with hot summer

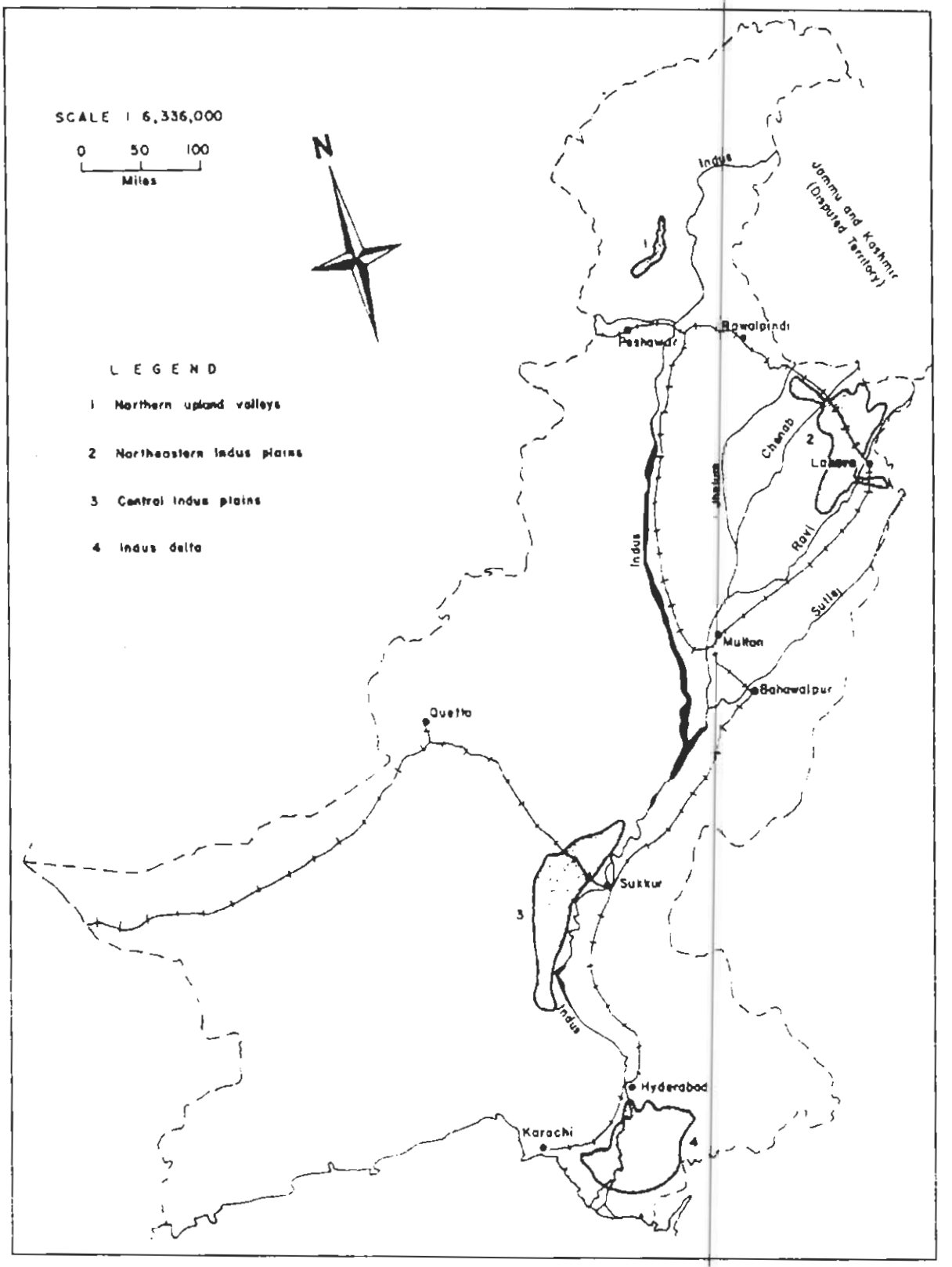

Fig. 3. Rice zones of Pakistan (Source, Chaudhry 1978).

with midday temperature 35-40°C and moderately cold winter. The mean temperature for October is 15°C and 10°C for November. The temperature regimes are favourable for one of the world's finest rice variety i.e. local aromatic Basmati variety. Due to the very high price, in spite of a lower the yield (1.2 t/ha) than IRRI varieties (1.9 t/ha), Basmati is sown in 80% of the rice area in this zone.

Previously rice was grown in these soil but for the last 40 years, due to availability of pumped ground water and common use of fertilizer, cropping has intensified and the rice crop is followed by wheat in about 50% of the area and 5% by winter clover. The crop of rice is grown by transplanting seedlings in standing water in June and first half of July, harvested in October and November. After rice harvest, wheat is sown in late November to December and is harvested in mid April to mid-May (Chaudh-ry 1978),

Rice has been grown in the Indus plain for centuries and certain soil management practices suitable in local conditions, have been evolved by farmers, and under the old system farmyard manure was applied once in 3 or 4 years, but presently most of the farmers apply 65 kg N/ha as urea or ammonium sulfate. Only a minority of farmers use about 50-60 kg P_2O_5/ha in addition to nitrogenous fertilizers. These soils are deficient in nitrogen and phosphorus but in some cases there is also a response to potash when added with N and P fertilizer. Zinc deficiency has also been reported in partly reclaimed saline sodic soils (Chaudhry, 1978).

Collection of *Azolla* and Water Samples:

To know the distribution, ecology and seasonal variation of *Azolla* population, a survey of rice growing area of the Punjab was carried out for two years in the months of January, February, May, June, October, November and December. The rice growing area situated mainly between Ravi and Chenab river, lying between 73-74.5 E, longitude and 31-32.5 N, latitude comprising Gujranwala, Sheikhupura, Sialkot and Faisalabad districts was surveyed. The surveyed area was located along the roads connecting, Faisalabad-Satiana, Faisalabad-Jaranwala-Bhai Pheru-Lahore, Faisalabad-Sheikhupura-Lahore, Sheikhupura-Muridke, Lahore-Muridke-Gujranwala, Sheikhupura-Gujranwala, Gujranwala-Daska-Sialkot, Sialkot-Sombrhial-Wazirabad, and Wazirabad-Gujranwala-Hafizabad-Chinyot-Faisalabad (Fig. 4).

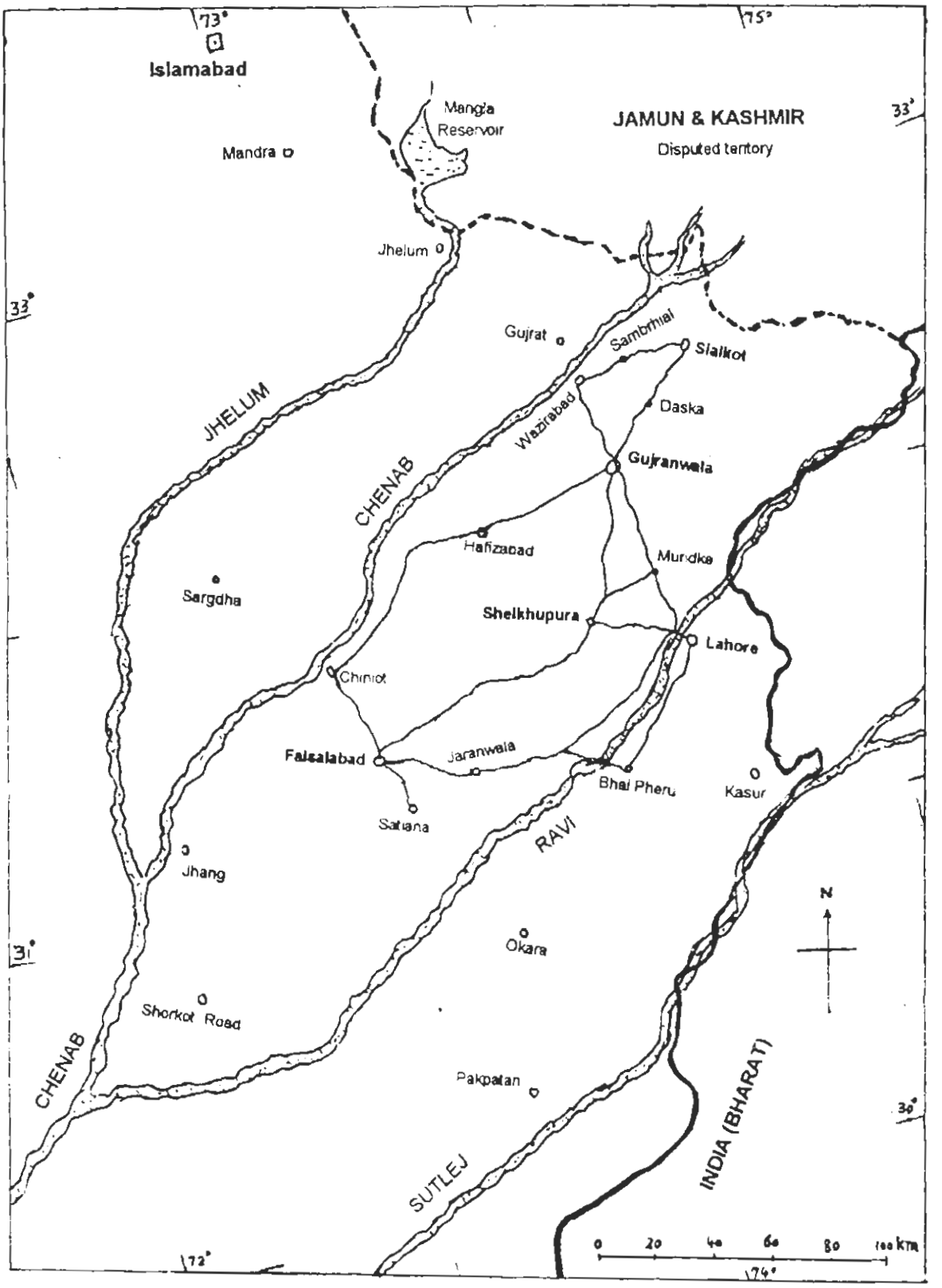

Fig. 4. Rice area travelled by road for *Azolla* survey in Punjab.

During this survey fresh water ponds, ditches, slow-moving-water drains, and rice fields along the roadside were observed for occurrence of *Azolla* and other plants growing along with *Azolla*. Since the exact quantification of *Azolla* floating on the water surface and measurement of irregular low-land area is practically very difficult, therefore to assess and compare *Azolla* growth at different places, percent area covered by *Azolla* out of the total water surface of a particular habitat was recorded. The condition of *Azolla* growth like patchy or full bloom, and health and colour of *Azolla* was also noted.

During this survey, 10 samples of *Azolla* plants were collected from six different places located in Gujranwala, Sheikhupura and Sialkot Districts for cultivation at Faisalabad. The plants were collected in small (500 ml) wide-mouth plastic bottles, containing 1-2 cm layer of original water. The plants were brought to Faisalabad and were cultured in different conditions to select the heat tolerant strains and maintain the nursery for laboratory, green house and field studies.

To know the effect of pH, EC and different nutrients on *Azolla* in field condition, floodwater samples from different places, having poor, medium and very good growth, were collected during the *Azolla* survey. The water samples were stored in cold room (10-15°C) prior to analysis, and chemical analyse. for EC, pH, cations like Ca^{++}, Mg^{++}, K^{+}/Na^{+}, and anions like CO_3^{--}, HCO_3^{-}, Cl^{-}, SO_4^{--} and $H_2PO_4^{-}$ was performed according to Richard (1954). Flame photometer was used for K^{+} and Na^{+} determination. The organic matter was determined by colorimetric procedure after wet digestion of sample according to CSTPA (1980) and micronutrients were determined with atomic absorption spectrophotometer (Yoshida et al. 1976).

TEMPERATURE STUDIES OF *AZOLLA*

The *Azolla* plants growing naturally in rice area, were brought to Faisalabad during winter and were cultured in soil-water medium. These plants died in May/June due to heat sensitivity. In the coming winter, *Azolla* plants from different places were again brought to Faisalabad and efforts were made to adapt and select the heat tolerant strain. During severe summer maximum temperature may rise to 47°C and relative humidity may fall to 16% at Faisalabad (Fig. 5). Therefore, during summer, ten different local strains of Azolla, collected from six sites of rice growing area, were cultivated in the green house under shade

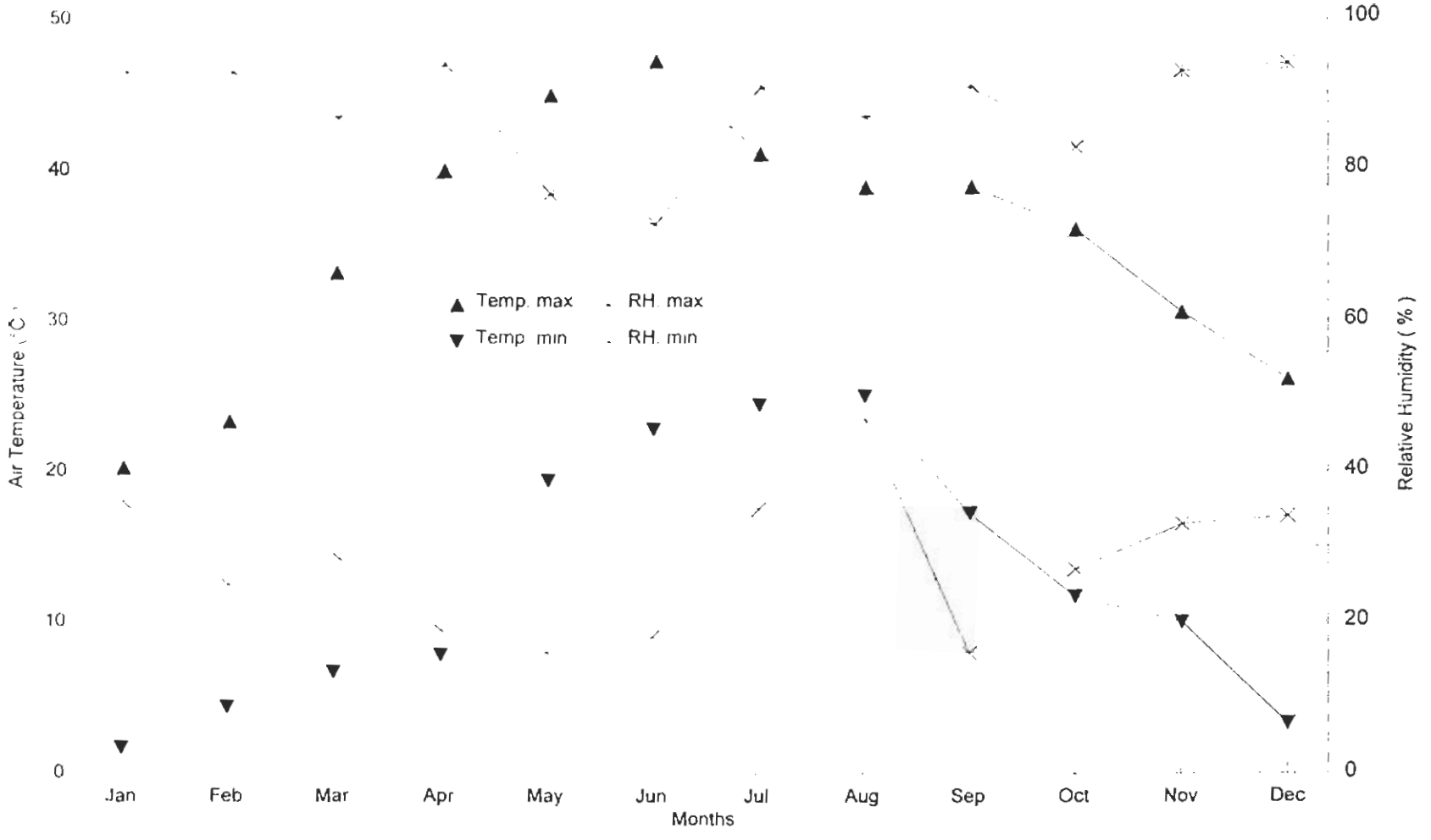

Fig. 5. Weather data of Faisalabad, 1994. (Data: courtsey of A. Kauser, NIAB).

and ice was also added to cool the culture solution at noon time. These strains were grown in small plastic sieves, which were placed in a large tank (2X1m) having a layer of about 5cm of a good soil and about 200L of canal water. To avoid any nutritional stress farmyard manure (10g/L) and superphosphate (10mg P/L) were also added to soil water culture, and Furadan (0.5g cabofuran/m^2) was applied for pest control.

The one strain which survived during severe summer was identified as *A. pinnata* tentatively and for confirmation of identification and culture collection, it was handed over to Dr. Iwao Watanabe, Head, Soil Microbiology Department, International Rice Research Institute, (IRRI), Los Banos, Laguna, Philippines as facilities and expertise for identification were available there, and was identified as *A. pinnata* var. *imbricata* (A.p.i).

To know the effect of temperature on *Azolla*, the local heat tolerant strain (A.p.i) selected after the above studies, was used. The green house experiment was performed during January-February as water temperature was controllable in water baths. Some water baths were multi-opening type having concentric rings for hole adjustment. The other baths, having no such openings, were fitted with a sheet of thermopore (synthetic heat insulating material) having holes of the size of the experimental beakers. Thus water of these baths was not exposed to ambient cold air and it remained almost constant as monitored with maximum-minimum thermometer during the experiment. Since the beakers of control set were not heated their temperature varied between 10 and 20°C. with an average temperature of 15°C. In addition to control (average temperature 15°C) four constant temperatures viz. 20, 25, 30 and 36°C of culture medium were maintained in the water baths. One liter polyethylene beakers, containing 900 ml of KB medium (Ali and Malik 1987) were placed in the holes of the water bath and there were four replicate for each temperature. The above said *A. pinnata* local was inoculated at the rate of 3 g fresh weight per beaker and the plants were exposed to natural sunlight of 60 K Lux (average light) and 11 hours day (7 am-6 pm) and 13 hour night regime.

After ten days of incubation at different temperatures, *Azolla* plants were collected in small strainers and excess of water was allowed to drain for about 10-15 minutes and fresh weight was recorded.

Since in nature, *Azolla* plants are likely to be exposed to high temperature along with some nutritional stress. Therefore, to know the effect of temperature on *Azolla* under such

stress condition, 3g of *Azolla*/pot was again inoculated into the same beakers, having relatively exhausted culture medium. After ten days of incubation, *Azolla* was harvested and fresh weight was recorded, as before.

During this temperature study, observations for morphology of *Azolla* plants and sporocarp formation were also made. The growth value (GV= fresh wt. of harvested plants/fresh wt. inoculum) and doubling time (DT=t log 2/log GV, when t= duration in days) were computed according to Kitoh and Shiomi (1991).

AZOLLA CULTIVATION

Azolla Cultivation in Defined Culture Media:

Biomass, chlorophyll content and ARA: To maintain the local *Azolla* under conditions resembling its natural habitat, four culture media were reconstituted according to floodwater analysis, after balancing the cations with anions by hit and trial method. Details of balancing of cations with anions and computation of salt concentrations for KB medium are given in Table 2a,b. Similarly, salts and their concentration were computed for other culture media, and four culture media were reconstituted according to water analysis, in which *Azolla* growth was better. As best growth was observed in floodwater of Muridke (MD), Khorhi site A and B, (KA and KB), and water of the black tub (BT) having soil+farmyard manure (FYM) in the green house, therefore these culture media were tried for *Azolla* cultivation (Table 3). *Azolla* medium of IRRI (Watanabe et al 1977) adjusted to pH 6.5 and 8.0 was also included to compare with the local culture media. The pH of all the 4 local culture media was adjusted to 8.0, as most of the floodwater of rice area had pH around 8.

For *Azolla* cultivation, plastic pots containing one liter of any of the above mentioned culture media were used and placed in green house. The experiment was performed during March (first week) to April (2nd week) when the average temperature was 25°C, sunlight 62 K lux and photoperiod 12.5 hours. One gram fresh (0.2 g dry wt) *A. pinnata* was inoculated per pot, and pH was reset and volume of culture media was made to original level, twice a week during incubation period. After 35 days of incubation *Azolla* plants were harvested and plants were dried at 70°C for recording the dry wt.

Table 2a. Balancing of cations and anions of floodwater analysis of Khorhi-B (KB) site to find the salt concentration.

Anion	Ca^{++}	Mg^{++}	Na^{+}	K^{+}	Total anion (meq/L).
HCO_3^-	-	-	2.0	-	2.00
Cl^-	0.335	0.515	1.0	0.115	1.965
SO_4^{--}	-	0.650	-	-	0.650
H_2PO_4	-	-	-	0.035	0.035
Total Cations (Meq/L)	0.335	1.165	3.0	0.150	4.650**

* Concentration of Cl- was reduced from 2.0 meq/L (as per analysis to this value to compensate for H_2PO_4.
** Total of all cations or anions.

Table 2b. Concentration of salt, based on balancing of ions, for reconstitution of K.B. culture medium.

Salt	meq/L*	mg/L**
$CaCl_2$	0.335	19.7
$MgCl_2$	0.515	24.5
$MgSO_4$	0.650	39.0
$NaHCO_3$	2.000	168.0
NaCl	1.000	58.5
KCl	0.115	8.6
KH_2PO_4	0.035	4.8

* Salt (meq/L) = Ion (meq/L).
** Salt (mg/L) = (Salt meq/L) X salt mol.wt/cation valence.

Table 3. Ingredients of Different Culture Media for *Azolla*[*].

Ingredients	Az.M.	BT	MD	KB	KA
		Macronutrients (g/liter)			
$CaCl_2$	0.333	0.139	0.036	0.019	0.019
$MgCl_2.6H_2O$	-	-	0.085	0.056	0.058
$MgSO_4.6H_2O$	0.492	1.185	0.141	0.076	0.111
$NaHCO_3$	-	0.252	0.365	0.168	0.252
KCl	-	0.073	-	0.008	0.008
K_2SO_4	0.274	-	-	-	-
Na_2SO_4	-	0.541	-	-	-
K_2CO_3	-	-	0.041	-	-
NaCl	-	0.148	-	-	-
$MgCO_3$	-	-	0.036	-	-
KH_2PO_4	-	0.005	0.005	0.005	0.005
NaH_2PO_4	0.12	-	-	-	-
		Micronutrients (mg/liter)			
Fe	0.2	0.16	0.09	0.63	0.09
Mn	0.1	0.03	0.03	0.06	0.03
Zn	0.012	0.02	0.02	0.02	0.02
Cu	0.005	0.003	0.01	0.01	0.01
Co	-	0.1	0.1	0.1	0.1
Mo	0.005	0.01	0.01	0.01	0.01
B	0.635	0.1	0.1	0.1	0.1

[*]Az.M = *Azolla* medium (Watanabe et al., 1977); BT = Black tub floodwater; MD = Muridke Drain floodwater; KB = Khorhi floodwater, site B; KA = Khorhi floodwater, site A.

At the time of harvesting of *Azolla* 0.2 g(fwt) was taken for chlorophyll extraction. The chlorophyll was extracted in 80% acetone and absorption was measured at 652 nm for total chlorophyll estimation (Yoshida et al. 1976).

On the last day, when *Azolla* was harvested, and there was no carrying effect of previous mineral nutrition, nitrogenase activity was measured for the above mentioned culture media. Since acetylene reduction is good measure of nitrogenase activity (Li et al 1987, Watanabe et al.1977, Turner and Gibson. 1980), therefore acetylene reduction assay (ARA) was performed using a simple set up. For ARA a glass incubation tube, 3 cm dia 25 cm height was placed in a beaker so that 10-11 *Azolla* plants (0.5-0.6 g fwt) were enclosed in the tube (Fig. 6). The level of respective culture medium in the beaker was adjusted to 100 cc mark of the incubation tube and a suba seal was fitted into the upper opening of the tube. Then with the help of a syringe, 10 cc air was withdrawn and 10 cc. acetylene was injected into the incubation tube for ARA and to keep the pressure unchanged. Similar set up, but without *Azolla* was used as control, and there were 3 replicates for each culture medium. After 2 and 3 hours of incubation in sunlight and 23 hours (day + night), gas samples from the incubation tubes were collected in vacutainers for analysis on gas chromatograph for ethylene production.

The *Azolla* plants enclosed inside the incubation tube were removed, slightly pressed between blotting paper and tissue paper to remove excess water and fresh weight was recorded. For chlorophyll estimation 0.2 g fresh *Azolla* was taken and chlorophyll was measured as mentioned above.

Effect of Humic Acid on Growth and N_2-Fixation: As farmyard (FYM) manure is generally used to enhance *Azolla* growth in the field, and humic acid is a major component of FYM, therefore, their effect on *Azolla* growth and nitrogenase activity was studied. For this purpose K.B. culture medium was used and *Azolla* was grown in green house in pots containing 1L of culture medium. Humic acid extracted according to Schnitzer (1982) containing 0.052% humic acid-C was added into this N-free KB medium at the rate of 0.0175,0.035 and 0.052% (V/V). The light and temperature conditions were similar as for *Azolla* cultivation in defined culture media.

A. pinnata @ 0.5 g fwt. was inoculated per pot and was allowed to multiply for 24 days and then was harvested to record fresh weight after removing the excess water with

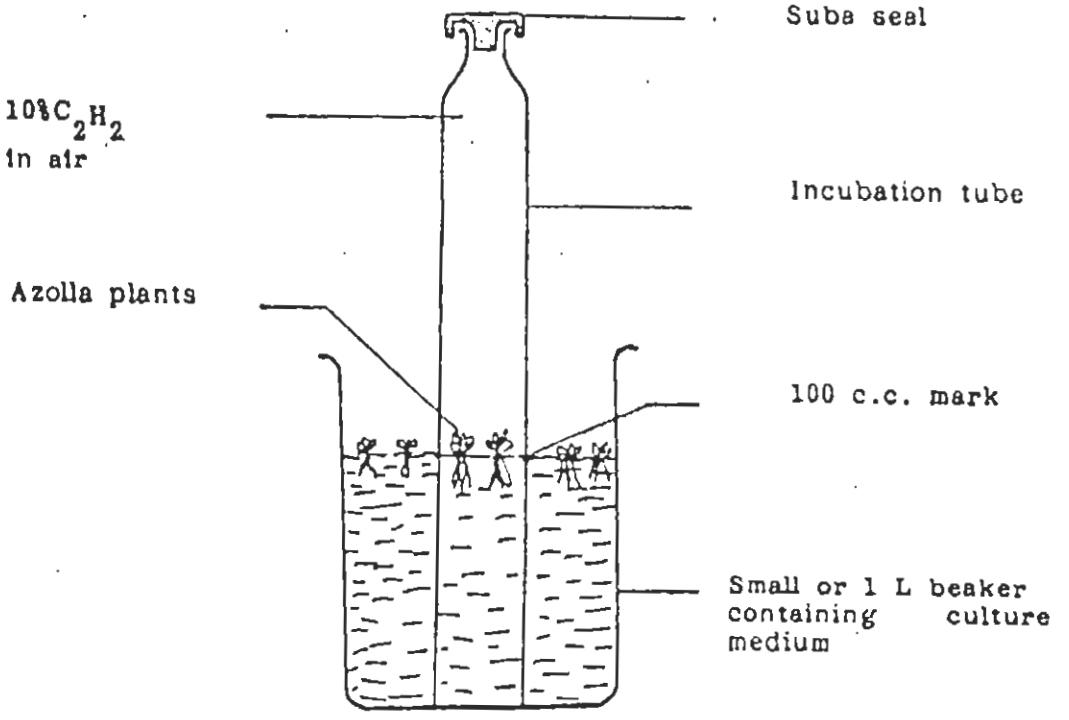

Fig. 6. A simple set for acetylene reduction assay of *Azolla*.

blotting and tissue paper. On the last day, before harvesting Azolla, ARA was measured as mentioned above. The growth value and doubling time were completed according to Kitoh and Shiomi (1991).

Azolla Cultivation in Undefined Culture Media:

As undefined culture media are required for maintenance of *Azolla* in green house and field conditions therefore different culture media were tried for *Azolla* cultivation.

Preliminary Experiment: To investigate the effect of soil, type of water, cowdung and some micronutrients, a preliminary experiment was performed in a green house. The sunlight was about 65 KLux and average temperature about 28°C and photoperiod of 13 hours. In plastic pots, 1 liter of medium was used and following treatments were used in 3 replicates:

T1 = Pondwater + pond soil + cowdung (All local material).

T2 = Khorhi floodwater + Local Faisalabad soil.

T3 = Local canal water + Mn + Fe.

T4 = BG-11, N free medium.

In case of T1, black soil of pond was placed at the bottom of the pot to make 1-2 cm thick layer, and after addition of pondwater, cowdung 0.1% of water (w/v) was also added. In case of T2, 1-2 cm thick layer of local non-saline, good soil was placed at the bottom, and original floodwater brought from Khorhi (rice growing area) during survey was added. In T3, local canal water having EC = 0.5 dS/m and pH 8.2 was used and concentration of Mn and Fe were made as in BG11 culture medium used for blue-green algae (Stainier et al. 1971), while in T4 N-free BG11 medium was used. Five *Azolla* plants (equivalent to normal size) were inoculated, and after one month of multiplication, plants were counted in each pot. For plant counting, each normal sized plants was counted as one, while two of ½ of normal sized plants as one and three plants of 1/3 of normal size as one. The counting of *Azolla* plants for an estimates of biomass is quite reliable and is also a non-disturbing and non-destructive method (Ali and Watanabe, 1986).

Comparison of Defined and Undefined Culture Media: To compare growth of *Azolla*, two defined and two undefined culture media were used. Plastic tubs (30 cm. dia.) three-

fourth filled with culture solution were used and four replicates were prepared for each of the following treatments:

1) KB culture medium, in distilled water (pH 8.0)

2) IRRI *Azolla* Medium, in distilled water (pH 6.5)

3) Farmyard manure (0.5%) in canal water (pH 8.5)

4) Local soil+0.5% farmyard manure+canal water(pH 8.0)

In case of T4, 1-2 cm layer of local good soil was placed at the bottom and 0.5% FYM (w/v) was used. The *Azolla* cultivation period was almost the same as that of rice, as it started on 15th August and ended on 15th December. One gram fresh *Azolla* was inoculated and the culture tubs were placed in green house where average daily air temperature was 30, 25, 20, 18, 15°C in August, September, October, November and December (Fig. 5), and the photoperiod (day length) was 14.5, 12.5, 11.5, 11.0 and 10.0 hours, respectively.

After 4 months of *Azolla* cultivation in the tubs, plants were collected in strainers, allowed to drain, and then pressed between blotting and tissue paper to remove extra water and fresh weight was recorded and doubling time was computed according to Kitoh and Shiomi (1991).

In the above said conditions, another pot experiment having three treatments with three replicates was conducted to compare the *Azolla* growth and nitrogenase activity. For this purpose 0.5g fresh *Azolla* was inoculated into 1L of one of the three culture media viz., KB, 0.5% FYM and 5% soil + 0.5% FYM. One day before harvesting, ARA was measured as explained previously. After 24 days, *Azolla* was harvested and fresh weight was recorded.

Long Period Cultivation in Soil-Water Culture: The long period sustainability of soil-canal water culture amended with different nutrients, on *Azolla* growth was studied in green house. Glazed pots containing 500g (air dried) paddy soil, and 13 liters of canal water having EC 0.25 dS/m, pH 8 and Ca^{++} + Mg^{++} 1.5, Na^{+} 0.2, K^{+} 0.08, CO_3^{--} 0.0, HCO_3^{-} 1.7, Cl^{-} 0.5 and residual sodium carbonate 0.25meq/L, were used for maintenance of *Azolla* nursery. Following 10 treatments were compared using 3 replicates:

1. Control (canal water)
2. Fe (2 mg/liter) as $FeCl_3.6H_2O$

3. P (10 mg/liter) as NaH_2PO_4
4. Fe + P (as in T2 and T3)
5. Micronutrients (as in IRRI medium, Watanabe et al. 1977)
6. IRRI *Azolla* medium (Watanabe et al. 1977)
7. Farmyard manure (FYM) @10g air dried/liter
8. FYM + Fe (as in T7 & T2 respectively)
9. FYM + P (as in T7 & T3 respectively)
10. FYM + Fe + P (as in T7, T2 & T3 respectively)

After addition of above said nutrients into pot water, 3 g fresh *Azolla* was inoculated in the beginning of second week of April (9th April) and *Azolla* was harvested at 3-4 week intervals and each pot was reinoculated with 3g *Azolla*, till the harvesting ended in last week of October (21st October). After each harvest excess of water was removed as in the above experiment and fresh wight was recorded and cumulative *Azolla* yield per pot was computed for the 26 week cultivation period.

Diagnosis of Nutritional Constraints: As some nutritional constraints are likely to occur for *Azolla* cultivation in the field conditions, therefore, a green house study was undertaken to know the nutritional problems like P, Zn and Fe in a soil to be used for rice-*Azolla* experiment. Plastic pots of 1.5 L capacity having soil water ratio of 1:5 (w/v) were used in this study. The pots were placed in natural light (avg. 60 K lux), maximum temperature 28-30°C and pH of pot water varied between 8-10 during day time, and floodwater EC 0.6-0.9 dS/m during *Azolla* growth period. Using missing nutrient technique of Watnabe et al (1977) the following 5 treatments with 3 replicates were used.

1) -P -Zn -Fe (Control, no fertilizer)
2) -P (+Zn +Fe)
3) -Zn (+P +Fe)
4) -Fe (+P +Zn)
5) +P +Zn +Fe (having all the three fertilizers).

In these pots P at 20 kg P_2O_5/ha (as super phosphate), Zn at 2 Kg Zn/ha (as $ZnSO_4.7H_2O$), and Fe at 0.5 kg Fe/ha (as $FeSO_4.7H_2O$) were applied on area basis. *A. pinnata var. imbricata* (the local heat tolerant) *Azolla* strain was inoculated at 0.2 g fresh

wt./pot and after 4 weeks of incubation plants were harvested and fresh and dry weight (at 70°C) was recorded.

One day before harvesting, ARA was measured as detailed in *'Azolla* cultivation in defined culture media'.

On the basis of above experiment, P was found to be the most common limiting nutrient for *Azolla* growth, therefore another experiment was set up using same soil and procedure, to find the optimum dose of P for *Azolla* growth. Five levels of P_2O_5/ha in 3 replicates were used:

T1 = 0P (control, no P fertilizer)

T2 = 10 kg P_2O_5/ha

T3 = 20 kg P_2O_5/ha

T4 = 30 kg P_2O_5/ha

T5 = 10 kg P_2O_5, in 4 split doses

In case of T2 to T4, superphosphate was applied once at the time of *Azolla* inoculation, while in T5 it was applied in 4 splits of 2.5 kg P_2O_5 at seven-day interval. After 4 weeks of multiplication *Azolla* was harvested and dry biomass was recorded.

PEST MANAGEMENT

Pests and their Control:

During cultivation of *Azolla* in green house, one of the problem encountered was that some water snails of family Lymnaeidae and Planorbidae belonging to Gastropoda, and small bivalve crustaceans belonging to Ostrocoda and water beetle (*Nymphula* sp.) ate up *Azolla* plants within a few days, and maintenance of *Azolla* nursery was badly affected.

To kill water snails of *Lymnaea* species (family Lymnaeidae) which were more abundant in soil-water cultures of *Azolla*, different concentrations based on active ingredient (a.i.), the following insecticides commonly used by farmers for crop protection were tested. The basic information about these five pesticides is given below:

1) Furadan (Carbofuran = 2-3-dihydro-2, 2-dimethyl-7 benzofuranyl methylcarbamate): Broadspectrum systemic (absorbed and translocated in the plant) insecticide, acaricide and nematicide. Usual dose 7.5-10 Kg of

'Furadan 3G'(3% a.i.)/acre and once per rice season is sufficient (CPCR 1987).

2) Nuvacron (Monocrotophos, 200g/L): Effective against wide range of pests including leaf-eating beetles, caterpillars, and phytotoxic to some plants under cool conditions (Hill 1975).
3) Heptachlor (Heptachlor, chlorinated hydrocarbon): Non- systemic, broadspectrum, used against different insects, beetle larvae etc. (Hill 1975).
4) Akar (Chlorobenzilate, emulsion): Used as acaricide, timing 2 week (RSC, 1984).
5) Endrin (Chlorinated hydrocarbon): Broad spectrum and persistent, more effective against insects biting with mouth parts than sap-suckers, neurotoxic (Hill 1975).

To find the minimum concentration of these insecticides on snails, *Lymnaea* species, were used. Different concentration viz. 2.5, 3, 5, 10, 20 and 30 ppm (a.i.) of each of the above said insecticides were prepared and 10 mature snails of almost equal size were put into 1 liter beaker containing insecticide. After 24 hours, live snails in each pot were counted to know the LD90 (Lethal dose for killing 90% of organisms).

Effect of Pesticides on *Azolla* Growth:

After the above experiment, the minimum concentrations required to kill snails (LD90) were tested for any adverse effect on *Azolla* growth. For this purpose 1 litre pots were used, and following concentrations on a.i. basis of each of these insecticides were made:

T1 = Control (Soil + water)
T2 = Furadan (30 ppm)
T3 = Nuvacron (30 ppm)
T4 = Heptachlor (30 ppm)
T5 = Akar (5 ppm)
T6 = Endrin (5 ppm)
T6 = Endrin (5 ppm)

The pots were placed in green house and 0.5g fresh *A. pinnata* was inoculated, and after 3 weeks of multiplication it was harvested and fresh weight was recorded after removing the excess water.

NITROGEN FIXATION IN *AZOLLA*

Nitrogen Fixation in the Presence of Combined N:

To investigate the effect of combined nitrogen on *Azolla* growth and nitrogen fixation a green house study was conducted. The following treatments were conducted in 3 replicates:

T1 = Control (N-free KB culture medium)

T2 = 14 ppm N

T3 = 28 ppm N

T4 = 42 ppm N

In case of T2 to T4, ammonium sulphate was used to make the desired level of concentration in 1L KB culture medium in plastic containers. *A.pinnata* @ 0.5 g fwt/pot was inoculated and after 24 days of incubation, fresh biomass was recorded. The cultivation conditions and ARA method were same as described previously under '*Azolla* cultivation' in defined culture medium.

Estimation of N_2 Fixation by ^{15}N Dilution Technique:

For ^{15}N dilution and estimation of N_2-fixation, ^{15}N enriched soil (used for ^{15}N labelling of *Azolla*) was used in this experiment. The soil had pH 7.6, EC_e 0.36 dS/m, organic C 0.8 % total N 0.086% (C:N 9.3) and exchangeable NH_4-N 5.5 ppm. The *Azolla* nursery plot (3x1.2 m) was divided into 4 blocks and was flooded to 1-4 cm water depth. The five *Azolla* species used in this study were, *A. pinnata* var. *imbricata* (local), *A. caroliniana*-301 and *A. microphylla*-418 (from culture collection in Thailand), and *A. caroliniana* and Rong Ping (hybrid *Azolla* from *Azolla* Research Centre, Fuzhon, Fujian, China. Plastic sieves (25 cm dia) having each of the 5 different species of *Azolla* inoculated at 6g (fwt.)/sieve were placed in floodwater in 4 replicates. Similarly 6 g/ of *Lemna minor* per sieve was also placed in the same plot. In addition to *Azolla* and *Lemna*, rice @ 100 seeds/sieve alone and alongwith *Azolla* was also grown in plastic sieves, in the same plot.

Due to perforations and weight of sieve they did not float but settled onto mud. The experiment was done in June/July, being the transplanting time of rice, and plants were harvested after 3 weeks of cultivation. Total N in *Azolla, Lemna* and rice at the time of inoculation and after harvest were determined by Kjeldahl method ^{15}N was estimated with mass spectrometry by the International Atomic Energy Agency at Vienna, under the *Azolla* project grant No. 3665. The nitrogen fixation was computed according to (IAEA, 1983) after correcting the ^{15}N abundance as suggested by Watanabe et al. (1991), using the following formulae:

$\%NdfN_2 = 1-\{(\%^{15}N \text{ a.e. Azolla}^{*}/ \%^{15}N \text{ a.e. non-fixer}^{*}) \times 100\}$

*Corrected ^{15}N a.e. = N in harvested plants/N in inoculum X % ^{15}N a.e. in harvested plants.

CARBON AND NITROGEN MINERALIZATION

Decomposition of *Azolla* in Soil:

The useful nutrients including fixed N becomes available to rice or other plant after the decomposition and mineralization of *Azolla* plant. A laboratory experiment was conducted to study the decomposition and mineralization of *Azolla* at two moisture regimes i.e. 30% and 100% water saturation of soil, simulating upland and lowland rice soil conditions respectively.

Decomposition of Unlabelled *Azolla*: A laboratory study was conducted to investigate the decomposition of *Azolla* by measurement of CO_2 evolved during this process in upland and lowland conditions. The rice soil used in this experiment was collected from Institute's paddy fields, and its water saturation percentage was computed after making saturation paste (Richard 1954) and pH of this paste was also recorded. The soil had 36% saturation percentage (36 ml water for 100 g of soil) and pH 8.0, total N 0.06% and organic C 0.466%. Air dried soil @ 250 g was added into 500 ml flask. For this study the following four treatments in triplicate were used:

T1 = Soil at 30% of water saturation percentage ($\overset{S}{\hat{P}}$)

T2 = Soil at 30% $S\hat{P}$ + *Azolla*

T3 = Soil at 100% SP

T4 = Soil at 100% SP+ *Azolla*

For *Azolla* treatment, 25 g fresh (2.38 g dwt.) *Azolla pinnata* var. *imbricata* (local) was mixed into soil. The *Azolla* had 3.47% N and 50% organic-C on dry weight basis. Distilled water was added to the flasks to obtain 30% SP moisture level (27 ml/250g soil) equivalent to 60% of water holding capacity, and to get 100% SP (90ml/250g soil)in the flasks. The flasks were stoppered with rubber bung having two glass tubing i.e. one connected to incoming air and the other to CO_2 receiver. The experimental flask were attached to aeration assembly, where a gentle stream of CO_2 free (by passing through 3N-NaOH solution in a 250 ml flask) and moist (then passing through distilled water) was passed over the soil and any CO_2 evolved from the soil was absorbed in a test tube containing 10 ml of 3N, NaOH acting as CO_2 receiver. The prevailing temperature in lab was 25-32°C during incubation period of 40 days.

The amount of CO_2 evolved was estimated titrimetrically (Anderson 1982) after different periods of incubation and each time CO_2 receiving test tube containing NaOH was replaced with a fresh one for next estimation. The net CO_2 evolution for *Azolla* treated soil was computed by subtracting the CO_2 evolved from control soil.

Decomposition of ^{14}C-Labelled *Azolla*: For uniform labelling of *Azolla* a closed system delivering ^{14}C labelled $^{14}CO_2$ for photosynthesis by *Azolla* was set up. *A. pinnata* was grown in a growth chamber with a 30±5°C temperature, 80% relative humidity and 9 K lux light for 16 hours a day. Pulse labelling of *Azolla* for 3 hours was done twice at 2-day intervals in a 6-liter flask with 1 liter KB medium containing 0.05% humic acid and 5% ammonium sulphate. $^{14}CO_2$ was generated inside the flask by adding dilute lactic acid into $Na_2(^{14}CO_3)$ solution contained in a small beaker hung to the stopper inside the flask. The $^{14}CO_2$ generated in the flask was mixed inside the flask by pumping in and out with a two-way cadet pump. After one week of cultivation, the *Azolla* plants were harvested. The major portion of ^{14}C-labelled *Azolla* biomass was used for ^{14}C-mineralization and $^{14}CO_2$ evaluation estimation in the laboratory condition (as already mentioned above, for mineralization of unlabelled *Azolla*), while a small amount of labelled *Azolla* was used to estimate its radioactivity by combusting in a Packard Sample Oxidizer and measuring the activity in a Tricarb 3320 liquid scintillation counter.

Azolla-N Mineralization:

A rice soil collected from the campus, having saturation percentage of 36 (18% water holding capacity), pH 7.98, ECe 1.82 dS/m, organic-C 0.5%, and total N 0.058% was used for this laboratory study. Air dried soil @ 50 g/flask was added into 100 ml flask and two g fresh *A. pinnata* (100 mg dwt.) having 50% organic-C and 3.47% total N on dry weight basis, was mixed into soil, and following treatment with 3 replicate for each treatment were used:

T1 = Soil at 30% saturation percentage (SP)

T2 = Soil at 30% SP + *Azolla*

T3 = Soil at 100% SP

T4 = Soil at 100% SP + *Azolla*

Since soil had to be analyzed for mineral N (NH_4 and NO_3-N) at five intervals of times, therefore total 60 (12X5) flask were prepared for the above four treatments. One set of 12 flasks having all the 4 treatments (3 replicates) was removed after 3,7,10,18 and 25 days of incubation period. The flask were incubated at 30°C and desired moisture level was maintained during the experimental period of 25 days.

The mineral-N (available-N or exchangeable-N, predominantly NH_4 and NO_3-N) was extracted from soil with 2M. KCl solution. The KCl-Soil solution was steam distilled with MgO to liberate NH_4, and for NO_3 determination same sample was again distilled with MgO and Devarda alloy according to Keeney and Nelson (1982). The net mineral N for *Azolla* was computed after subtracting the amount of mineral-N produced in control.

Contribution of *Azolla* to Soil Humus:

After 40 days of incubation at 30 and 100% SP and CO_2 estimation from the soil of experiment of 'Decomposition of unlabelled *Azolla*' the same soil was analyzed for determination of humus fractions. The soil from the control (no *Azolla*) and from soil+unlabelled *Azolla* flasks, was taken out. It was air dried and ground to pass through 2 mm sieve.

The humin, humic acid and fulvic acid fractions of humus formed in soil were extracted according to Schnitzer (1982). The total N in humic and fulvic acid and in soil were estimated by Microjheldahl method (Bremner and Mulvaney, 1982), while organic

carbon using rapid dichromate oxidation method using sulphuric acid + $K_2Cr_2O_7$ for digestion and heating at 110°C for 1.5 hour and measuring the absorbance of green colour of Cr^{3+} at 600 nm (Malik et al 1979, Nelson and Sommers, 1982).

USE OF *AZOLLA* IN RICE

All the green house and field plot experiments were conducted at the campus of Nuclear Institute for Agriculture and Biology (NIAB) and National Institute for Biotechnology and Genetic Engineering (NIBGE) at Faisalabad. The climate of Faisalabad is dry for most of the months of the year except during rainy season of monsoon (July-August), and is very hot and dry during summer (April-June) and quite cold during winter as already shown in Fig. 5. In rice-wheat cropping system, rice is grown during summer and wheat in winter.

The rice nursery is grown in about 1/40 of the area to be transplanted. The nursery of IRRI varieties like IR-6 is mostly sown from May 20 to first week of June while of local tall varieties like Basmati-370 from first week of June to first week of July. About one month old seedlings of rice are transplanted in the 3rd week of June to Ist week of August, and the crop is harvested in first week of November to mid-December.

To investigate the effect of the local heat tolerant *Azolla pinnata* var. *imbricata* (A.p.i) on rice and subsequent wheat crop various experiments were performed under green house and field conditions. To know its effect on tall as well as short stature varieties; Basmati-370 which is fine grain aromatic, high priced and most commonly grown by the farmers, while IR-6 the popular IRRI variety, were used respectively. The first three green house and the first two field experiments were performed for Basmati-370, while the remaining studies were carried out for IR-6 rice variety. In the IR-6 experiments ^{15}N labelled nitrogen fertilizer and/or ^{15}N labelled *Azolla* was used to trace nitrogen recovery in rice and/or wheat, and for computation of their N use efficiency in rice-wheat cropping system.

Greenhouse Studies:

Effect of *Azolla* and Blue-green Algae (Pot Expt. 1): For this experiment, paddy soil from rice growing area of the Institute having sandy loam to sandy clay loam soil, was

Azolla growing as intercrop with rice (Greenhouse studies).

collected from the top 30 cm of different fields. The soil was thoroughly mixed and passed through 2 mm sieve and filled into 25 cm diameter glazed ceramic pots at the rate of 10 kg(air dry) per pot to make about 25 cm depth in the pot. The mixed soil, used in this experiment was sandy loam (sand 62, silt 22 and clay 16%) having electrical conductivity of saturation extract (ECe) 2.5 dS/m, pH_e 8.3, $CaCO_3$ 3%, organic matter 0.54 %, and total N 0.065%. The soil in the pots was flooded with canal water, and mixed with stick to simulate puddling conditions of rice fields. The pots were kept flooded until 2 weeks before rice harvest.

In the last week of August, one-month-old seedlings of rice cv. Basmati-370, were transplanted at five hills and 2 seedlings per hill, thus making total 10 seedlings per pot i.e. 2 in center and 8 at four sides. After four days of transplanting (DAT) of rice, a starter dose of nitrogen @ 10 kg N/ha (as urea) on area basis was applied as solution onto floodwater of all the plots. The following eight treatments with five replicates in randomized complete block design (RCBD) were performed in this study.

T1. Control (No fertilizer or biofertilizer).

T2. *Azolla* Intercropped and Incorporated: After 8 DAT, *Azolla* was inoculated @ 15 g fresh weight (fwt)/pot [0.7 g dry weight (dwt), having 25 mg N] equal to inoculum of 3 metric ton (t) fwt/ha (5 kg N/ha) on area basis. Whenever it covered the whole water surface, about one-half was incorporated into the soil by hands and the remaining half was left as inoculum for multiplication for next incorporation. Thus *Azolla* was incorporated on 15, 30, 37, 43, 54 and 70 DAT (total 6 incorporations) during the rice growth period

T3. *Azolla* Intercropped and Incorporated + Culture Medium: After inoculation of *Azolla* as in T2, N-free IRRI *Azolla* culture medium (Watanabe et al 1977) @ one liter per pot was added once, and the incorporations were made as in T2.

T4. *Azolla* Intercropped and Incorporated + Culture Medium + 2t/ha Farmyard Manure (FYM): Same as T2, but 10 g FYM (dwt) per pot (2t(dwt)/ha) was broadcasted after one day of *Azolla* inoculation.

T5. Dead *Azolla* + Culture Medium + FYM: Same as T4, but instead of live *Azolla*, heat-killed dead *Azolla* (by heating the water containing *Azolla*) equal to live inoculum was added to the pot water.

T6. Blue-green algae (BGA) + Culture Medium: A mixture of heterocystous (nitrogen fixing) blue-green algae, comprising mainly *Anabaena, Calothrix* and *Tolypothrix* sp. was inoculated @ 10 ml/pot (0.1 mg N/pot) 8 DAT. These BGA were previously isolated (Ali and Sandhu 1976) from the saline soils (Ali and Sandhu 1972) and rice soils (Ali et al. 1978) and were grown in N-free BG11 culture medium (Stanier et al. 1971) in laboratory conditions. After algal inoculation one litre of N-free BG11 culture medium was added per pot.

T7. BGA + 40 kg N/ha: After algal inoculation as in T6, a dilute solution of urea was added onto floodwater 25 DAT, at 40 kg N/ha (on area basis), once.

T8. BGA + 60 kg N/ha: Heat killed BGA equal to inoculum, was added onto floodwater 8 DAT, and urea solution as in T7 was applied 25 DAT, only once during rice growth period.

After 54 days of rice transplantation, attack of stem borer insects occurred, therefore Furadan (3% Carbofuran) was applied @ 1.5 g/pot (10 kg a.i./ha).

The agronomic parameters like flag leaf area, second leaf area, fertile and sterile tillers were recorded between 60-65 DAT. The leaf area was computed according to Yoshida et al. (1976) after measuring the length and width of the respective leaves. The maximum tiller height and panicle length were measured at maturity i.e. one week before harvesting.

Rice was harvested, at the end of the 3rd week of December, by cutting 1-2 cm from the soil surface. Grains from straw were separated and were dried at 60°C for dry biomass recording. After rice harvest, soil sample from each pot were taken with sampling tube for total nitrogen estimation by micro-Kjeldahl method (Bremmer and Mulvaney, 1982).

Effect of *Azolla* and Urea (Pot Expt. 2): For this experiment, the rice soil of last year's pot experiment No.1, was taken out of the pots and after thoroughly mixing, was put into 25 cm. diameter, but bigger (deeper) pots @ 12 kg (air dry)/pot. Like previous pot experiment, one-month-old seedlings of Basmati-370 were transplanted at 5 hills/pot and 2 seedling/hill in the first week of August. Following 8 treatments with 4 replicates in RCBD were performed.

T1. Control (no fertilizer or biofertilizer).

T2. *Azolla* Intercropped and Cover: Like previous experiment A.p.i (local strain) was inoculated, but @ 10g fwt pot equal to 2 t/ha (3.3 kg N/ha) at 7 DAT. In

this treatment, *Azolla* was allowed to grow and was not incorporated, but left as cover during the whole period of rice growth.

T3. *Azolla* Intercropped and Incorporated: Inoculated *Azolla* as in T2 and was grown as intercrop, and about one-half was incorporated and the remaining left as inoculum for multiplication for next incorporation as detailed in previous experiment.

T4. *Azolla* Intercropped and Cover+FYM (12 t/ha): After one day of inoculation of *Azolla* as in T2, FYM @ 12 t(dwt)/ha was broadcasted onto *Azolla*/floodwater.

T5. *Azolla* Intercropped and Incorporated+FYM: As in T4, but *Azolla* was incorporated as in T3.

T6. Dead *Azolla*: Heat killed *Azolla* (in hot water) equal to live inoculum was added into floodwater of the pot.

T7. 10 Kg N/ha: Urea @ 10 kg N/ha (area basis) was sprinkled in a dilute solution on 25 DAT and *Azolla* was incorporated as in T3.

T8. 50 Kg N/ha: Urea dilute solution was sprinkled onto floodwater @ 50 kg N/ha on 25 DAT.

Like the previous pot experiment No.1, rice was harvested at maturity during Ist week of December and was dried at 60°C for biomass recording.

After harvesting rice, wheat was sown to see the residual effect of *Azolla* on the subsequent crop.

Effect of *Azolla* and Farmyard Manure (Pot Expt. 3): After the rice and wheat harvest of pot experiment No.2, the soil was taken out of pots, mixed and refilled into pots @ 11 kg/pot. Like previous experiments, Basmati-370 was transplanted in the first week of July @ 2 seedlings/hills and 5 hills/pot. Following 8 treatments with 4 replicates were performed in RCBD.

T1. Control(without fertilizer or biofertilizer).

T2. *Azolla* Intercropped and Cover: Like previous experiment 10 g fresh A.p.i. per pot equal to 2t/ha (3.4 kg N/ha) was inoculated 15 DAT, and was allowed to grow as cover.

T3. *Azolla* Intercropped and Incorporated: As in T2, but about one-half was incorporated when full cover, and remaining was left for multiplication for next incorporation. Thus total four incorporation being on 21, 35, 48 and 58 DAT were made during rice growth period.

T4. FYM (6 t/ha): FYM was broadcasted onto floodwater 16 DAT.

T5. *Azolla* Intercropped and Incorporated+FYM: After *Azolla* inoculation as in T2, FYM @ 6 t/ha was applied onto *Azolla* and pot water as in T4, and *Azolla* incorporation were made like T3.

T6. *Azolla* Intercropped and Incorporated+P: After *Azolla* inoculation like T2, superphosphate @ 60 kg P_2O_5/ha (area basis) was sprinkled as suspension onto *Azolla*/floodwater, and *Azolla* was incorporated as in T3.

T7. Dead *Azolla*: Fresh *Azolla* equal to inoculum was killed by heating (in the water) and put into pots.

T8. 60 kg N/ha: Urea @ 60 kg N/ha (area basis) was applied in solution form onto floodwater on 25 DAT.

Due to insect attack on *Azolla*, Furadan (3% Carbofuran) was applied @ 10 kg ai/ha 35 DAT to all the pots. At maturity rice was harvested and dried at 70°C for estimation of straw and grain biomass, as mentioned for pot experiment 1.

Effect of *Azolla* on Fertilizer-N Use Efficiency (Pot Expt. 4): For this green house experiment, paddy soil was collected from the rice fields of the Institute. The soil was loam (14% clay, 33% silt and 53% sand), having pH (1:1) 7.4, EC (1:1) 0.95 dS/m, organic-C 0.49% and total N 0.067%. After air drying and sieving through 2mm sieve, 10 kg soil was added into 25 cm diameter ceramic glazed pots. Soil was flooded for two week and puddled for two weeks before transplanting of rice, one-month-old seedlings (5 leaf stage) of rice cv. IR-6 were transplanted @ two seedlings per hills and five hills per pot, in the last week of July. The pot soil was kept flooded (1-4 cm) throughout the rice season and watering was stopped 2 weeks before harvesting the rice. The following nine treatments with three replicates in RCBD were performed.

T1. Control (no N fertilizer, no *Azolla*).

T2. *Azolla* Intercropped and Cover: *Azolla pinnata* var. *imbricata* (already mentioned under pot experiment. 1) was inoculated @ 15g fwt (0.888 dwt) per

pot equal to 300 g/m^2 or 3 t/ha (fwt) 20 DAT of rice. It was allowed to grow, during rice growth period without incorporation into soil.

T3. *Azolla* Intercropped and Incorporated: As in T2 grown as intercrop, but about half was incorporated whenever it covered the water surface during rice growth, as mentioned for pot experiment 1. Before *Azolla* incorporation, floodwater was not drained from the pots to avoid ^{15}N losses, but watering of pots was stopped about 3 days prior to each A*zolla* incorporation by which water level decreased to 0.5-1 cm due to evapo-transpiration. Total seven incorporations of *Azolla* were made being 31, 39, 46, 56, 60, 68 and 81 DAT.

T4. 30 kg N/ha: ^{15}N labelled urea having 1.972% ^{15}N atom excess (a.e.) was applied @ 30 kg N/ha (area basis) in a dilute solution form onto floodwater 23 DAT.

T5. 60 Kg N/ha: ^{15}N labelled urea once as in T4, and second time again @ 30 kg N/ha 70 DAT, making a total 60 kg N/ha.

T6. *Azolla* Intercropped and Cover + 30 kg N/ha: *Azolla* as in T2 and urea like T4.

T7. *Azolla* Intercropped and Cover + 60 kg N/ha: *Azolla* as in T2, while urea as in T5.

T8. *Azolla* Intercropped and Incorporated + 30 kg N/ha: *Azolla* as in T3, and urea like T4.

T9. *Azolla* Intercropped and Incorporated + 60 kg N/ha: *Azolla* as in T3, while urea as in T5.

To enhance Azolla growth, a few hours before *Azolla* inoculation, superphosphate in the form of suspension was sprinkled onto floodwater @ 40 kg P_2O_5/ha into *Azolla* as well as in all the other pots. *Azolla* samples for total N and ^{15}N determination were collected 80 DAT from all the *Azolla* applied pots.

The pH of floodwater was measured with a portable pH meter *in situ*, and also with a precise laboratory pH meter by taking the water samples on 11 different days (between 56 and 111 DAT) at different times of the day (8 am to 4 pm) during rice growth period. After recording pH in the laboratory, the water samples were returned back to the respective pots to avoid ^{15}N losses from the system. The pH at soil-water interface and top 3-5 cm soil, was

measured at 80, 89 and 93 DAT. The temperature of floodwater and top 3-5 cm soil was measured at noon on 70, 74, 82, and 90 DAT. The algal growth was observed in floodwater and on soil surface on 30, 54, and 80 DAT.

The data for tiller number and maximum tiller height were recorded 3 days before harvesting, and at maturity rice was harvested at the end of November. Straw and grain were separated manually and were dried at 60°C and dry biomass was recorded. The dried sample of straw and grain were ground in a self cleaning mill (Cyclotec) to avoid sample loss and 15N cross contamination. After determination of total N with Kjeldahl method (Bremner and Mulvany, 1982), the distillate was acidified and evaporated to 1-2 ml and was used for 15N determination with a mass spectrometer (Hauk 1982), and ^{15}N calculations were made according to IAEA (1983).

Field Studies:

Effect of *Azolla* and urea (Field Expt. 1): The field experiment was conducted in the rice area of the Institute in 2x3 m plots, arranged in four blocks, in randomized complete block design (RCBD), having five treatments with one replicate in each block. The soil was sandy loam, having ECe 1.11, pH 8.1, Ca+Mg 12.5 me/L (saturation extract), organic 0.62% and total N 0.065%.

The plot area was prepared by ploughing and then divided into 20 plots in 4 blocks by making bunds. The soil was irrigated with canal water and puddled, and plots were kept flooded until 2 weeks before rice harvest. One-month-old seedlings of Basmati-370 were transplanted at 22x22 cm (9x9 inches) spacing, with one seedling per hill (9x13 hills per plot) during first week of August. The following five treatments with 4 replicates were performed.

T1. Control (no fertilizer or biofertilizer).

T2. *Azolla* Intercropped and Incorporated: After inoculation of *Azolla* @ 60g fwt/plot equal to 0.1t/ha (0.17 kg N/ha) on 20 DAT, it was allowed to multiply in the field to form a full cover and then approximately one-half was incorporated into soil by walking and mixing *Azolla* into soil with feet. For efficient incorporation of *Azolla*, instead of draining the plots, irrigation was stopped a few days prior to incorporation, so that 0-1 cm water was standing

Azolla growing as intercrop with rice (Field studies).

in the plots. After one day of *Azolla* incorporation, plots were irrigated, so that remaining *Azolla* plants floated up and multiplied in the field, and thus three incorporation were made during rice growth period.

T3. *Azolla* Intercropped and Incorporated + FYM (12 t/ha): After one day of *Azolla* inoculation as in T2, farmyard manure was broadcasted onto *Azolla* and floodwater @ 12 t/ha, and *Azolla* was incorporated as in T2.

T4. *Azolla* Intercropped and Incorporated + 10 kg N/ha: After *Azolla* inoculation as in T2, urea @ 10 kg N/ha was applied in solution form onto floodwater on 25 DAT, and *Azolla* was incorporated as in T2.

T5. 60 kg N/ha: Urea in the form of a dilute solution was applied 25 DAT, onto floodwater of the plots.

To have an estimate of amount of *Azolla* incorporated or standing as cover, fresh *Azolla* biomass was quantified by using a 0.25x0.25m quadrant on 50 DAT.

At maturity rice was harvested in the first week of November by cutting 1-5 cm above the soil surface with a sickle, leaving two boarder rows on each side. Fresh weight of straw was noted and sub-sample of straw while all the grain of each plot, were dried at 60°C for dry biomass estimation.

Effect of *Azolla* and Farmyard Manure (Field Expt. 2): After harvesting wheat, sown after rice of field experiment-1, the soil of the same plots was ploughed and puddeled, and Basmati-rice seedlings were transplanted at 22x22 cm in the first week of August, as detailed in field experiment-1, and the following five treatments with four replicates in RCBD were performed.

T1. Control (no fertilizer or biofertilizer).

T2. *Azolla* Intercropped and Incorporated: A.p.i.(local strain) @ 60 g fwt/plot equal to 0.1 t/ha (0.18 kg N/ha) being 50 g/plot was inoculated at 30 DAT and then again 10 g/plot at 50 DAT. As mentioned in the field experiment 1, about half of the total *Azolla* was incorporated with feet and half was left as inoculum, and total three incorporation were made during rice growth period.

T3. Farmyard Manure (6 t/ha): Farmyard manure @ 6 t/ha was broadcasted 10 DAT onto floodwater of plots.

T4. 60 Kg N/ha: Urea was applied, in a dilute solution form, onto floodwater 25 DAT.

To encourage *Azolla* growth superphosphate @ 60 Kg P_2O_5/ha was applied 28 DAT to Azolla as well as to all the other plots.

At maturity rice was harvested in the first week of November and biomass of straw and grain dried at 60°C was estimated as detailed for field experiment 1.

Evaluation of *Azolla*-N Uptake and Yield Response (Field Expt. 3): To measure and compare the availability of N from N-labelled *Azolla* and urea to rice and their effect on rice, a pot experiment was conducted according to the plan of isotope plot experiment (Expt.1b) of International Atomic Energy Agency (IAEA), Vienna, Austria, Contract No. 3665/RB.

Preparation of ^{15}N labelled *Azolla*: A 10 m^2 nursery plot having wide and strong bund was made for production of ^{15}N labelled *Azolla*. To avoid leakage of ^{15}N material from the nursery, a polyethylene sheet was placed in the plot and soil was spread to make about 10 cm thick layer on the sheet. As suggested in the experimental plan of IAEA Expt. 1b, the nursery area was divided into half and then one side half was further divided into two halves, with smaller and removal ridges of soil. One quarter of the nursery was flooded to a depth of 4-5 cm, and 1200 g fresh *Azolla* was inoculated by mid-July. After *Azolla* inoculation, 5 g of 10% ^{15}N a.e. urea in one litre was sprinkled onto *Azolla*/floodwater, and 5.153g superphosphate (0.2g P/m^2) was also applied to this nursery portion. After 3 days (of *Azolla* inoculation) labelled urea was again applied. After 5 days, the next quarter was flooded and ridge (between 2 quarters) was removed, and 10g of labelled urea dissolved in 2 litres water was applied to the nursery, and a low dose (3.8g) Furadan granules (containing 3% carbofuran) was applied onto *Azolla* nursery. On 8th day 10g labelled urea was again added and superphosphate (10.3g) was also applied. After 12 days, the remaining half of nursery was flooded and ridge was removed and *Azolla* was dispersed over the whole water surface. On the same day, 20 g labelled urea, dissolved in 4L of water was sprinkled onto *Azolla* nursery and superphosphate (20.6g) was also applied. After 15 days, the application of urea and P was repeated again.

For the second time of *Azolla* incorporation, ^{15}N labeled *Azolla* was again prepared during the last week of September to 2nd week of October in the same manner.

Unlabelled Azolla was grown in the same type of nursery but without adding any nitrogen fertilizer.

Three days before inoculation of *Azolla* into experimental pot (and plots), three samples of *Azolla*, 10 g fwt each; were taken for dry weight and total N estimation, so that amount of *Azolla* could be computed to supply 30 kg N/ha.

Isotope Plot Experiment: For this experiment, soil in the rice area was selected. All the isotope plot measuring 1x1 m were separated from each other with 30-35 cm bunds. To prevent movement of ^{15}N between plots, a continuous sheet of polyethylene plastic was lined towards the inner side of each bund and an overlap of about 25 cm was made. The plastic sheet extended vertically to a depth of about 20-25 cm below the soil surface and upward about 30 cm onto inner side of bund. There were 4 blocks, and each block had six plots. Thus 6 treatments, with 4 replicates were arranged in RCBD.

The soil was flooded and puddled and then superphosphate @ 45 kg P^2O^5/ha was broadcasted onto water and soil was again puddled. One-month-old seedlings (5 leaf stage of IR-6 were transplanted in the 3rd week of July at 20x20 cm spacing @ 2 seedlings per hill (50 seedling at 25 hills/plot) leaving 10 cm between the outer side plants and the bunds of the plot.

The following 6 treatments with 4 replicates were performed.

T1. Control: No nitrogen, no *Azolla*: but simulated incorporation.

T2. *Azolla*, $30^{15}N+30^{14}N$: Fresh ^{15}N labelled *Azolla* having 3.064% a.e. and 0.266% N on first basis was incorporated @ 30 kg N/ha at 40 DAT, and then unlabelled *Azolla* containing 0.184% N (fwt.) supplying also 30 kg N/ha was incorporated at 80 DAT.

T3. *Azolla*, $30^{14}N+30^{15}N$: Fresh unlabelled *Azolla* (0.196% N (fwt.) containing 30 kg N/ha was incorporated 40 DAT, while ^{15}N labelled *Azolla* having 5.743% a.e. and 0.268% N fwt. supplying the same amount of N was incorporated 80 DAT.

T4. Urea, $30^{15}N+30^{14}N$: ^{15}N labelled urea (1.972% ^{15}N a.e.) applied in the form of solution onto mud and then incorporated at 40 DAT @ 30 kg N/ha, and again the same amount at was incorporated 80 DAT but of unlabelled urea.

T5. Urea, $30^{14}N+30^{15}N$: Unlabelled urea @ 30 kg N/ha incorporated at 40 DAT and same amount of ^{15}N labelled urea (1.972% a.e.) at 80 DAT in solution form onto mud and then incorporated into soil.

T6. Urea, $20^{15}N+40^{15}N$: ^{15}N labelled urea (0.800% a.e.) both times, being 20 kg N/ha at 40 DAT and then 40 kg N/ha at 80 DAT applied in solution form and incorporated into mud.

Before *Azolla* or urea incorporations, floodwater was not drained to avoid any N losses but water was allowed to dry to mud stage in the plots, so that there was no standing-water present on the soil surface. *Azolla* or urea solution was spread evenly over the surface of mud, and then it was thoroughly mixed into soil with feet. Simulation of incorporations was made in control plots at the time of *Azolla*/urea incorporation. After one day of *Azolla* incorporation, plots were flooded again and any plants coming to surface were reburied into soil.

The rice was grown in flooded conditions until 10-15 days before harvest. One day before harvesting, maximum tiller height, total and fertile tiller of central 9 hills were recorded. At maturity, out of the total 25 hills, central 9 hills were harvested in the last week of November, while leaving the outermost row as boarder. The panicles were cut from the straw, then straw and panicles were dried at 60°C. The dried composite samples of straw and panicle for each replicate were ground to 40 mesh and samples were sent to FAO/IAEA Seibersdorf Laboratory in Vienna for ^{15}N determination using Dumas combustion method with mass spectrometer. The total N was determined by micro-Kjeldahl method.

After rice harvest soil samples (0-15 and 15-30 cm depth) from each plots were collected for total N and ^{15}N analysis and bulk density was measured using cylinder method.

During rice growth period, soil temperature at 5 cm depth was n oted with the help of a maximum/minimum thermometer, and average temperature was computed from these readings.

Evaluation of *Azolla*-N Uptake and Yield Response (Field Expt. 4): To confirm the results of the last year Field Experiment 3, the same treatments of isotope plot were repeated.

^{15}N labelled *Azolla* was prepared in the nursery plot of the previous year in the same manner (pot experiment 5), but using ^{15}N labelled ammonium sulphate (50% ^{15}N a.e.), from

mid July to Ist week of August for the first incorporation, while from last week of August to 3rd week of September for the second incorporation.

Isotope Plot Experiment: To avoid error of previous ^{15}N in the soil new area for this rice experiment was selected. Like last year, 1x1 m isotope plots were prepared, lined with polyethylene and IR-6 was transplanted in the first week of August. The same 6 treatment and same *Azolla* and urea material as for field experiment 3 were performed in the same manner, however, time of *Azolla* or urea incorporation was different (earlier than previous Expt.) as given below.

T1. Control: (as in above Field Experiment 3).

T2. *Azolla*, $30^{15}N+30^{14}N$: ^{15}N labelled *Azolla* having 1.082% a.e., and 0.1477% N on fwt. basis was incorporated @ 30 kg N/ha at 10 DAT, while the unlabelled *Azolla* having 0.205% N on fwt. basis was incorporated @ 30 Kg N/ha at 40 DAT.

T3. *Azolla*, $30^{14}N+30^{15}N$: Unlabeled *Azolla* having 0.1925% N (fwt.) was incorporated at 10 DAT, then ^{15}N labelled *Azolla* having 20716 a.e., and 0.224% N (fwt.) at 40 DAT, supplying 30 kg N/ha each time, were incorporated.

T4. Urea, $30^{15}N+30^{14}N$: ^{15}N labelled urea having 10.73% a.e. @ 30 kg N/ha at 10 DAT, while unlabelled one, at the same rate at 40 DAT were incorporated.

T5. Urea, $30^{14}N+30^{15}N$: Application as in T4, but unlabelled urea first time while labelled one (10.73% a.e.) for second time.

T6. Urea $20^{15}N+40^{14}N$: Both time ^{15}N labelled urea (10.73% a.e.), being 20 kg N/ha at 10 DAT, while 40 kg N/ha at 80 DAT were incorporated into mud.

The procedure for incorporation of *Azolla* and urea was the same as for the last year's field experiment 3. Maximum tiller height and total tiller for central 9 hills were recorded one day before rice harvest. The central 9 hills were harvested in the first week of December, and fresh and dry biomass and nitrogen analysis were done as described for previous field experiment 3.

Effect of P Enriched *Azolla* on N Availability (Field Expt. 5): To determine the effect of different levels of P fertilization on *Azolla* growth, N accumulation and its N

availability on rice, a field experiment was conducted mainly according to Experiment No. 5 of IAEA Contract 3665/RB.

Phosphorus Bioassay: To select a P responsing soil for *Azolla* growth, 7 different soils from the rice area of the Institute were bioassayed, so that *Azolla* with different %N could be prepared. For this green house test 500g soil/pot was added and one litre canal water was added to fill the pot, 2-3cm below the brim. *Azolla* was collected from the nursery, washed with water then with deionized water to removed soil adhering to plant/roots. These cleaned plants were grown in deionized water for 3 days to reduce carry over of P in the inoculum, and 10 plants were inoculated @ 1 g fwt/pot (75 g/m^2) during second week of July. For each soil there were control (no P application) and +P treatment and there were three replicates for each soil and treatment. Superphosphate @ 0.2g P/m^2 was applied at 0 and 7th day of *Azolla* inoculation. After 18 days of *Azolla* growth, plant were collected and after recording fresh weight, plants were dried at 60°C and dry weight was recorded.

^{15}N Labelling of *Azolla* at Different P Levels: Based on the results of the above bioassay, one soil was selected for growing *Azolla* at 4 different P levels. About 10 cm thick layer of this soil was spread on the polyethylene sheet in the 4 *Azolla* nursery plot each measuring 1.5x3m (4.5m^2). Each nursery plot was divided into two halves and one of the half was further divided into 2 portions, thus making 3 portions (¼, ¼ and ½ of original 4.5m^2 plot). One of the ¼ portion (quarter) of each plot was flooded with canal water (10-12 cm water depth) and fresh *Azolla* @200 g/m^2 was inoculated in the last week of August. To improve *Azolla* growth micronutrient solution was sprinkled on 10th day of *Azolla* inoculation, and two days later black mud collected form old nursery with good *Azolla* growth was added @ 1 kg/plot.

For ^{15}N labelling, 1.1g of 50% ^{15}N a.e. ammonium sulphate in one litre of water was dissolved and sprinkled on to *Azolla* of each quarters in each of the 4 nursery plots. After 5 days, the next quarter was flooded, ridge removed, and 2.12 g of labelled fertilizer dissolved in two litres of water was added to *Azolla* nursery, and the application of labelled fertilizer (2.1g/2L) was repeated on 8th day. After 10 days, the remaining half of each of the nursery plots was flooded, ridge removed, and *Azolla* was dispersed. Then 4.2 g of labelled fertilizer dissolved in 4 litre of water was applied to each of the 4 nursery plots and this application of labelled fertilizer (4.2 g 4L) was repeated on 12th day.

During the above mentioned ^{15}N labelling, different doses of P were also applied to *Azolla* nursery. The four levels designated as OP, 2P, 4P and 6P were formed as follows. For all the 4 levels superphosphate suspension was sprinkled onto *Azolla*/floodwater.

OP: No P application. *Azolla* to be used for T5.

2P: Two application of 0.2 g P/m^2 being first on 0 day (the day of *Azolla* inoculation) and on 5th day of *Azolla* inoculation. *Azolla* for T6.

4P: Four application of 0.2 g P/m^2, being on 0,3,5 and 8th day of *Azolla* inoculation. *Azolla* for T7.

6P: Six application of 0.2 g/m^2, being on 0,3,5,8,10 and 12th day of *Azolla* inoculation. *Azolla* for T8.

After completion of ^{15}N labelling at all the four P levels, samples of *Azolla* were collected for dry weight and %N determination, and for computation of N in *Azolla* biomass.

Water Sample for P Determination: To monitor the changes in floodwater P, during ^{15}N labelling of *Azolla* at four different P levels (0,2,4,6 P), water samples of 30 ml were collected daily from each nursery for determination of P concentration. On those days when superphosphate was applied, one sample was collected before P addition and the other one hour after P application. To stop microbial activity and facilitate filtration, samples were acidified to pH2 by adding 1.5ml conc. HCl (Ali and Watanabe 1986). After filtration the P in water was determined spectrophotometri-cally according to Olsen and Sommers (1982) method for P soluble in water.

The field experiment was conducted in 1x1 m isotope plots in the rice area of the Institute, and plots and soil were prepared as in isotope plot experiment of field experiment no. 3. Five-week-old seedlings of IR-6 were transplanted at 20x20 cm at the end of July. The *Azolla* grown at 0,2,4 and 6 P levels was used, and 8 treatment; were performed in this field study using 4 replicates in RCBD, as given below.

T1. Control: No nitrogen, no *Azolla*; but incorporation simulated.

T2. 15KgN/ha: ^{15}N labelled urea (10% ^{15}N a.e.), applied in solution form at 40 DAT and then incorporated into soil.

T3. 30Kg N/ha: Similar to T2, but @ 30 kg N/ha.

T4. 45 Kg N/ha: Similar T2, but @ 45 kg N/ha.

T5. *Azolla*, OP: ^{15}N labelled *Azolla* grown at OP in the nursery, incorporated @ 222.7g fwt (17.2964g dwt)/m^2 containing 2.081% N on dwt basis, at 40 DAT.

T6. *Azolla*, 2P: ^{15}N labelled *Azolla* grown in 2P nursery, containing 2.622% N was incorporated @ 311g fwt (15.446 g dwt)/m^2, at 40 DAT.

T7. *Azolla*, 4P: ^{15}N labelled *Azolla* grown in 4P nursery, containing 2.564% N was incorporated @ 266.7 g fwt (15.646 g dwt)/m^2 at 40 DAT.

T8. *Azolla*, 6P: ^{15}N labelled *Azolla* grown in 6P nursery, containing 2.749% N was incorporated @ 266.7 g fwt (17.336 g dwt)/m^2 at 40 DAT.

The procedure for incorporation of *Azolla*, harvesting of rice, sample grinding for total and ^{15}N analysis and sampling of soil after rice is the same as in isotope plots of field experiment no. 3.

Effect of *Azolla* on Urea ^{15}N Recovery (Field Expt. 6): To determine the effect of *Azolla* on rice yield, its N availability to rice and on the fertilizer-N recovery in rice and soil N budget, field experiment in isotope plots were performed according to the plan of IAEA experiment 7 of contract no. 3665/RB.

Isotope Plot Experiment: To avoid any interference of previously applied ^{15}N in to soil, new area from rice fields of the Institute was selected. Unlike previous isotope plot experiment having 1x1 m^2, the isotope plot area was reduced to 0.8x0.6m, to reduce the input of expensive ^{15}N labelled fertilizer.

^{15}N Labelling of *Azolla*: Since a small amount of ^{15}N labelled *Azolla* was needed due to smaller size of isotope plots, therefore a part of the old nursery area being (1x3m) was used. This nursery was divided into 2 halves and in one half 1kg *Azolla* was inoculated after 5-6 cm flooding and labelling of Azolla was initiated in the second week of July. For *Azolla* labelling, 1.8g of ^{15}N labelled ammonium sulphate (95% ^{15}N a.e.) dissolved in 1 litre of water and was sprinkled onto *Azolla*/floodwater at the time of inoculation. again on 3rd and 6th day of inoculation. After 8 days, the other half was flooded, ridge removed and after dispersing *Azolla*, solution of labelled ammonium sulphate having 3.6g in 3L of water was sprinkled onto *Azolla*. The application of 3.6g of labelled fertilizer, dissolved in 3L was again repeated on 10th and 13th day, after *Azolla* inoculation. During application of ^{15}N labelled fertilizer to *Azolla* nursery, superphosphate @ 0.2gP (0.46 P_2O_5)/m^2 suspended in 1L of water, was sprinkled onto *Azolla* mat on 0, 3, 6, 8, 10 and 13th day of *Azolla*

inoculation. Three days before its use in the experiment, total N was determined for computation of its amount for pot and field experiment.

The unlabelled *Azolla* was prepared in the same manner but without application of any nitrogen fertilizer in the adjacent area to the above said nursery, and its nitrogen was also determined for computation of its amount for the field experiment.

The method of soil preparation, lining with polyethylene sheet is the same as detailed under isotope plot experiment of field experiment 3. The 36 isotope plots (0.8x0.6m) were divided into 4 blocks, thus the following 9 treatments with 4 replicates were arranged in RCBD, and 6-week-old seedling of IR-6 were transplanted at 20x20 cm spacing (4x3 hills/plot) in first week of August.

T1. Control: (no nitrogen, no *Azolla*).

T2. 30 kg N/ha at Transplanting: ^{15}N labelled urea (20% a.e.) incorporated at one DAT.

T3. 30 Kg N/ha+*Azolla* Intercropped and Cover: Urea as in T2, *Azolla* inoculated @ 200 g fwt/m^2 (96g/plot) having 4.11% dry weight and 4.1% N, at 3 DAT.

T4. *Azolla* 30 Kg N/ha: ^{15}N labelled *Azolla* @ 800 fwt/plot (4.51% dwt & 4.114%N) containing 30 kg N/ha was incorporated at one DAT.

T5. 30 Kg N/ha: ^{15}N labelled urea (20% a.e.) added into floodwater 40 DAT (not incorporated).

T6. *Azolla* Intercropped and Cover+30 kg N/ha: Inoculated *Azolla* as in T3, while ^{15}N labelled urea @ 30 kg N/ha added into floodwater at 40 DAT (not incorporated).

T7. *Azolla* Intercropped and Incorporated+30 Kg N/ha: Urea addition and *Azolla* inoculated as in T6, but it was incorporated 60 DAT.

T8. 60 Kg N/ha: ^{15}N labelled urea (10% a.e.) incorporated one DAT.

T9. *Azolla* Intercropped and Cover: Inoculated *Azolla* as in T3 and it was allowed to grow without any incorporation, and no nitrogen was applied.

To enhance *Azolla* growth superphosphate was applied in suspension form at 0.2 g P_2O_5/plot in all the *Azolla and* other plots.

In case of *Azolla* cover treatment, in some plots continuous floodwater layer could not be maintained due to percolation in the early days after rice transplanting, therefore,

Azolla growth suffered, hence *Azolla* was reinoculated @ 500 g/plot in all the *Azolla* intercrop-treatment plots of T3, T6, T7 and T9 at 20 DAT i.e. after 2 weeks of first *Azolla* inoculation.

During this experiment, whenever *Azolla* was incorporated, simulation of incorporation was also done in all the other plots also. In addition to climate data, floodwater temperature and pH (as mentioned in pot experiment 9), soil temperature at 5 cm depth was also recorded during rice growth period.

The rice was grown in flooded conditions, till 2 weeks before harvest. At maturity, central 2 hills were harvested in the 3rd week of November, while outermost row on each side (total 10 hills) were left as boarder . The straw and panicle were separated and dried at 60°C for dry weight recording, and total N and ^{15}N analysis; as mentioned in field experiment no. 3.

After rice harvest, using cylinders of 28 mm (inner dia) X 150 mm length soil sample were collected for bulk density estimation. To collect root and soil sample, soil around 2 central hills (20x40 cm) was dug out from 0-15 cm and 15-30 cm depth separately. Rice roots were removed from the soil and dried for dry weight and ^{15}N analysis. The soil of top 0-15 cm and lower one 15-30 cm was mixed separately and subsamples were prepared for ^{15}N analysis.

RESIDUAL EFFECT OF *AZOLLA* ON WHEAT

As mentioned earlier, that in rice-wheat cropping system, rice is harvested in mid-November to mid-December and then wheat is sown in the same soil. After harvesting rice crop, the soil is allowed to dry to "Wattar" i.e. approximately to field capacity moisture stage and ploughed to prepare seed bed. The wheat is normally sown after second week of November till the end of December, and is harvested during the last two weeks of April or upto second week of May.

In the following greenhouse and field experiments, wheat was sown in the same soil, after harvesting tall variety as well as dwarf variety of rice. No fertilizer (N or P or K) and no biofertilizer like *Azolla*, and no compost or farmyard manure, was applied to soil after harvesting the rice.

To differentiate the experiment number of wheat from rice each of the pot and field experiment number follow the letter "R" indicating experiment for residual effect of *Azolla* on wheat. The first green house as well as the first field experiment is for the wheat grown, after tall variety of rice Basmati-370; while the remaining experiments are for wheat, grown after dwarf variety of rice IR-6.

Greenhouse Studies:

Pot Experiment 1R: To know the residual effect of *Azolla* and urea on wheat, wheat cv. Pak -81 was sown after harvesting the rice from pot experiment 2. After harvesting Basmati-370 rice, soil was allowed to dry to "Wattar" stage of moisture. The soil of each pot was mixed and prepared for sowing seeds, with a hole. Total 15 seeds of wheat cv. Pak-81, at the rate of 3 seeds per hole, and 5 holes per pot(1 in centre and 4 on sides),were sown in each pot in the 3rd week of December. After a week of emergence of wheat seedling, ten seedling of almost uniform size and distributed equally in the pot were kept for further growth and the remaining ones were removed and burried into the soil of the same pot. During wheat growth period weed were removed and burried back into the soil of the same pot. The pots were watered 4 times i.e. in the second week of February, and in the beginning, middle and end of March. In the last week of April, wheat plants were cut 1-3 cm above soil surface, and straw and grains were separated manually and dried at 60°C for dry biomass estimation.

Pot Experiment 2R: After harvesting the IR-6 rice of pot experiment 4, soil was prepared as in pot experiment 1-R, and wheat cv. Blue-Silver was sown at the end of December. The watering schedule and weeding was done as in pot experiment 1-R. At maturity wheat was harvested in the second week of May, and dry biomass of straw and grain was recorded after drying at 60°C.

Field Studies:

Field Experiment 1R: After harvesting the Basmati-370 rice from 2x3m plots of field experiment 1, wheat cv. Punjab-81 was sown in the same plots. Since at the time of rice harvest soil was wet therefore it was allowed to dry to "Wattar" (field capacity) stage being

considered the most suitable stage for ploughing and preparation of soil for sowing wheat. The soil was ploughed and prepared with farm tools and wheat seed were sown in 9 rows, length wise, with 22 cm distance between rows. To keep the plant papulation uniform, amount of seed at the rate of equal to 100 kg/ha was separately weighed and sown in each plot.

During wheat growth, irrigation were given as per normal practice. Thus plots were irrigated 4 times i.e. in the second week of February and in the beginning, middle and end of March, and weeds were removed and buried back into the soil of the same plot.

At maturity, wheat was reaped with a sickle and grains were separated from the straw with a small electric thresher. The straw and grain were dried at 60°C and dry weight was recorded.

Field Experiment 2R: After harvesting IR-6 rice from plots of field experiment 3, wheat cv. Blue-Silver was sown @ 100 kg seed/ha in the first week of December in isotope and yields plots.

In isotope plot of 1x1m size, 10g seeds were sown per plot, in 5 rows, having 20 cm distance between row to row for central rows, and 10 cm distance between bund to rows for the 2 outer most rows.

The hoeing and irrigation schedule was similar to field experiment 1-R. At maturity (second week of May), in case of isotope plots, central 3 rows (0.6x0.6m) were harvested and outermost one row was left as border. Straw and grain were separated as detailed in field experiment 1-R and dried for recording dry biomass and N analysis.

During wheat growth period soil temperature at 5 cm depth was also recorded as detailed in the rice field experiment no. 3.

Field Experiment 3R: After the rice harvest of field experiment 4, wheat cv. Blue-Silver was sown in both the isotope and yield plots in the 3rd week of December and was harvested in the 3rd week of May, as detailed in the above field experiment 2R.

Field Experiment 4R: After rice harvest of field Expt. 5, wheat cv. Pak-81 was sown in the second week of December in isotope plots and was harvested in the first week of May, as detailed in the above mentioned field experiment 2-R.

Field Experiment 5R: After harvesting the rice from the isotope plots of field Expt. 6, wheat cv. Pak-81 was sown in the first week of December and was harvested in the second week of May, as detailed in the field experiment 2-R.

RESULTS

AZOLLA SURVEY IN RICE AREA

Distribution and Ecology:

During the survey of rice growing areas of the Punjab, *Azolla* was found growing at 19 places (Table 4) and most of these sites (Site no. 5 to 18) were in the upper central Punjab. It grew mainly in stagnant or slow-moving water of drains, small ponds, and ditches (17 sites), while only in 2 rice fields, one being fallow and the other had Basmati rice crop (Site no.8 and 19). It was not found in fast moving canal water, rather in slow-moving water it was mainly present near the banks and among other plants.

The *Azolla* cover ranged from a very small patchy growth to a bloom condition covering quite a large area of water surface being up to about 500 m^2. The colour of *Azolla* varied from green (mostly in small patches) to dark green and reddish-green with large sized plants at the bloom sites. The other plants growing along with *Azolla* were *Lemna minor, Hydrilla verticellata, Typha angustata, Ipomea aquatica, Eichhornia* sp., *Leptochloa fusca* and different other grasses (Table 4).

The *Azolla* was found growing abundantly in the above mentioned places only during the moderately cold months i.e. mid-February to mid-March and October to November. The *Azolla* plants were slightly less frequent during severe winter (mid-December to mid-February) and almost disappeared during very hot months i.e. from May to mid-July, in most of the places.

Effect of Floodwater Properties on *Azolla* Growth:

The chemical analysis of floodwater and soil-water cultures (Table 5) indicated that *Azolla* grew in a wide range of nutrient concentration. The waters, in which *Azolla* was found growing, had pH 7.8-8.9, EC 0.3-2.3 dS/m, while cations like Ca^{++} 0.31-2.5, Mg^{++} 1.2-9.6, Na^{+} 2.3-15.8, K^{+} 0.14-2.77, and anions like CO_3^{--} 0-2, HCO_3^{-} 2-6, Cl^{-} 0.7-9, SO_4^{--} 0.4-17.3 meq/L and the P (as H_2PO_4 ion) in floodwater ranged from 0.05 to 0.2 ppm. The organic carbon ranged 1-135, Mn 0.03-2.93, Fe 0.06-0.25, while Zn and Cu from traces to 0.2 and 0.01 ppm respectively.

Table 4. Natural Occurrence of *Azolla pinnata* var. *imbricata* in the Rice Growing Areas of the Punjab.

S/No.	Site/ Location	Type of Water	Condition of *Azolla* growth	Other common plants in the habitat
1.	**Faisalabad City:** Pond on south of railway station.	Large pond +sewage water	Greenish plants on sides	Many greases, *Hydrilla,* blue-green algae
	Satiana Area			
2.	Chak No.80 G.B. 18 KM from Faisalabad	Water channels and ditches with stagnant water	Reddish-green plants	Green algae
3.	Satiana Bangla near Police Station	Ditches along main road side	-do-	Algae and grasses
4.	**Jaranwala-Bhai Pheru Road:** Near Buchaike	Ditches along road side	-do-	-do-
	Lahore-Sheikhupura Road:			
5.	3 KM from Sheikhupura	Stagnant water in low lying area	Greenish plants in large patches	*Marsilia, Lemna. Typha*
6.	11 KM from Sheikhupura	Ditches and small ponds	Reddish plants. patchy growth	-do-

	Sheikhupura-Muridkey Road:			
7.	Upper chenab canal area, 10 KM from Sheikhupura	Drain water	Reddish plants in bloom	*Hydrilla verticellata*
8.	Rice fields, 15-16 KM from Sheikhupura	Fallow rice field after rice harvest clay suspended water, soil black	Reddish plants good growth	Some young shoots from cut rice & v. few grasses
9.	Drain crossing the road at 17-18 KM from Sheikhupura	Stagnant drain water	Healthy reddish plants in bloom	*H. verticellata, Ceratophyllus*
10.	Ravi Rayon Factory Area, 22 KM from Lahore	Stagnant water in lowlying area	Greenish plants in small patches	*Lemna minor* dominant, Grasses
11.	Khorbi area, 31 KM from Lahore near "Pul Saumori" District Sheikhupura	Drain water 10 cm-1m deep	Reddish-green, large sized plants bloom at 2 places	*Eichchornia, Crassipes, Typha, Diplachne*
12.	Sadhoke Rail-way Station area, 35 KM from Lahore	Slow moving drain water	Healthy plants, bloom at one place	*Fusca, H. verticellata* & *Typha angustata*
13.	Drain, 37 KM from Lahore	Slow moving drain	Good growth	*H. verticellata, T. angustata. Prosopis juliforis* (Mosquito)

14.	Ditches along G.T. Road, 62 KM from Lahore	Ditches, small ponds	Patchy growth	*Ipomea aquatica*, grasses
	Gujranwala-Sialkot Road:			
15.	Nandipur area near village "Othian" about 13 KM from Gujranwala	Stagnant water in lowlying area	Green-reddish plants in large patches	*T. angustata*, *E. crassiper*, *I. carnea* and *Nymphaea* sp.
16.	Lowlying area, 15 KM from Gujranwala	Stagnant water in lowlying area	Rare and small patches	Grasses
	Sialkot-Wazirabad Road:			
17.	Mandair area, near village Mandair, 16 KM from Sialkot	20-59 cm stagnant water in lowlying area	Green-reddish plants, v. good growth	*I. carnea*, *H. verticellata* *Nymphaea* sp.
18.	Sambrhial area, about 16 KM from Sialkot	a) Ditches & ponds	Reddish plants, good growth	*H. verticellata*, *Typha* and Grasses
		b) Basmati Rice field, with mature rice plants	Greenish plants in patches	Rice plants, 1m tall, *H. verticellata*
	Faisalabad-Chiniot Road:			
19.	Drain a few KM from Chiniot	Drain, stagnant water	Reddish-green in patches	*Spirogyra*, Grasses

Table 5. Azolla cover and chemical analysis of flood water collected from rice growing area of the Punjab and green house soil-watre cultures.

Location	Az.C	pH	EC	Ca	Mg	Na	K	CO3	HCO3	Cl	SO4	Org.C	Mn	Fe	Zn	Cu
Sheikhupura-Muridke rice field	60	8.3	0.8	0.70	2.8	2.3	0.19	0.20	4.6	0.7	0.4	na	na	na	na	na
Sheikhupura-Muridke road, drain	90	8.3	1.1	0.65	2.9	4.4	0.24	0.30	5.1	1.5	1.2	na	na	na	na	na
Ravi Rayon factory area	20	8.9	1.8	0.16	1.6	15.8	0.43	2.00	2.0	9.0	4.9	4	0.04	0.09	0.01	0.003
Khorhi area -A	90	8.5	0.3	0.34	1.5	3.0	0.14	nil	3.0	1.0	0.9	1	0.03	0.09	nd	nd
Khorhi area -B	85	8.5	0.3	0.34	1.2	3.0	0.15	nil	2.0	2.0	0.7	1	0.06	0.06	nd	0.010
Khorhi area -C	80	8.7	0.7	0.70	2.5	6.8	0.90	1.00	4.0	2.0	3.9	6	0.43	0.22	nd	nd
Sadhoki Railway Station area -A	65	7.8	1.2	0.75	3.0	6.8	2.77	nil	6.0	5.0	1.5	135	2.93	0.25	0.02	nd
Sadhoki Railway Station area -B	60	8.1	1.0	0.75	2.7	6.0	1.50	nil	5.0	5.0	0.9	103	0.87	0.13	0.01	nd
Sadhoki Railway Station area -C	50	8.7	0.8	0.66	2.2	4.6	1.14	1.00	4.0	4.0	0.6	131	0.26	0.13	nd	0.010
Nandipur area	50	8.7	0.6	0.31	1.9	4.6	0.17	1.00	3.0	2.0	1.0	25	0.03	0.09	nd	nd
Sambrhial rice field	40	8.6	0.4	0.25	1.3	4.8	0.15	0.50	3.5	1.0	1.5	1	0.03	0.09	nd	0.010
Greenhouse pot	10	8.4	1.2	0.41	1.5	8.8	0.18	nil	3.0	1.5	6.4	1	0.03	0.09	nd	nd
Greenhouse tub	100	8.2	2.3	2.50	9.6	13.2	1.00	nil	3.0	6.0	17.3	50	0.03	0.16	nd	0.003

Az. C = Azolla cover(%); EC = Electrical conductivity(dS/m); Org.C = Organic Carbon(mg/L)
Cations & Anions(meq/L); Micronutrients(mg/L); na = not analyzed; nd = not detectable

To find the relationship of different floodwater properties to *Azolla* growth, which are not very clear from the above table 5, the enveloping graph technique of Balandreau and Ducerf (1980), suitable for field data, was used. Using the floodwater surface covered by *Azolla* as an indicator of *Azolla* growth, the relationship between *Azolla* growth and different chemical properties and /or constituents of floodwater is presented in Fig. 7. The floodwater pH less than 8.7 favoured *Azolla* growth, while at higher pH a lesser growth was observed (Fig. 7a). The lower EC i.e. 0.3-1.1 dS/m of floodwater had good effect on *Azolla* growth as compared to higher salt concentration (Fig. 7b). The Ca^{++}+Mg^{++} concentration seems to be of wider range for *Azolla* growth, as *Azolla* cover was equally good from 1.5 to 3.5 meq/L, however at higher concentration the *Azolla* growth may be retarded, carbonates, while a higher concentration, may have adverse effect on *Azolla* growth (Fig. 7d). Unlike carbonates, a concentration of bicarbonates from 2 to 5 meq/L was useful for *Azolla* growth while a higher concentration may not be desirable (Fig. 7e). Lower concentration of chlorides (1-2 meq/L) encouraged *Azolla* growth whereas higher values did not favour its growth (Fig. 7f). Like bicarbonates, a reasonable concentration of SO_4^{--} i.e. 1-4 meq/L was required for good *Azolla* growth, while at higher concentrations its growth was retarded (Fig. 7g). A wide range of organic matter in floodwater seems to be acceptable for *Azolla* growth as its cover was similar from 1 to 100 ppm concentration of floodwater organic-C (Fig. 7h). As regards the trace elements, desirable concentration of Fe was 0.05-0.2 ppm and of Cu from traces to 0.01 ppm (Fig. 7i & 7j). The Na:Ca and Na:K ratio, computed on meq/l basis, showed that a lower Na/Ca ratio (less than 10) favoured *Azolla* growth, while the Na/K ratio of 7-25 was desirable for *good Azolla* cover (Fig. 7k & 7l).

TEMPERATURE STUDIES OF *AZOLLA*

Selection of Heat-Tolerant Strain:

During survey of rice growing area, *Azolla* plants, collected from different sites had different colour and size, but when grown at Faisalabad in the green house, they showed a similar morphology. The 10 different local strains of *Azolla* collected from 6 different sites during *Azolla* survey of the rice growing area of the Punjab, behaved differently for growth in severe summer at Faisalabad. Seven died by the end of April, and 2 by the middle of

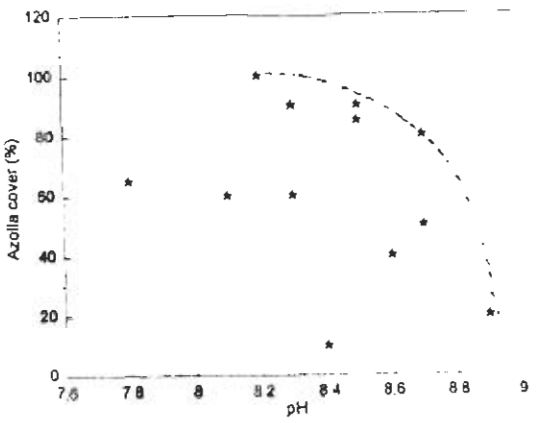

Fig.7a. Effect of floodwater pH on Azolla cover.

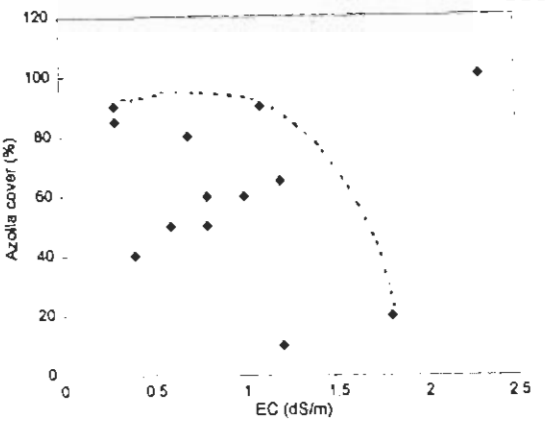

Fig.7b. Effect of floodwater Ec on Azolla cover.

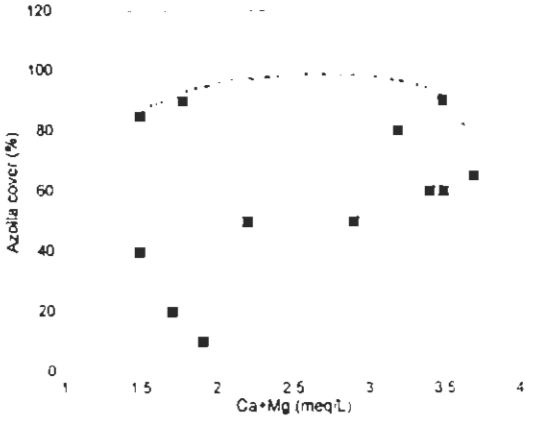

Fig.7c. Effect of floodwater Ca+Mg on Azolla cover

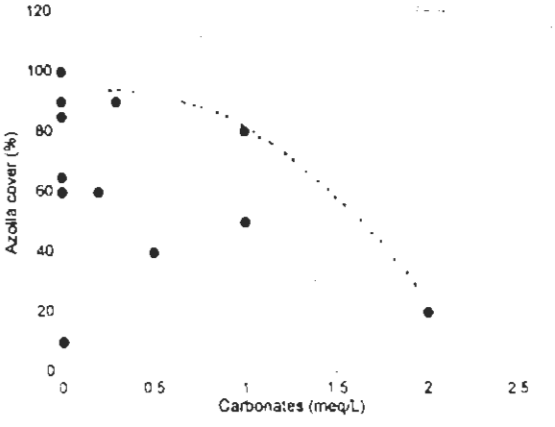

Fig.7d. Effect of floodwater carbonates on Azolla cover.

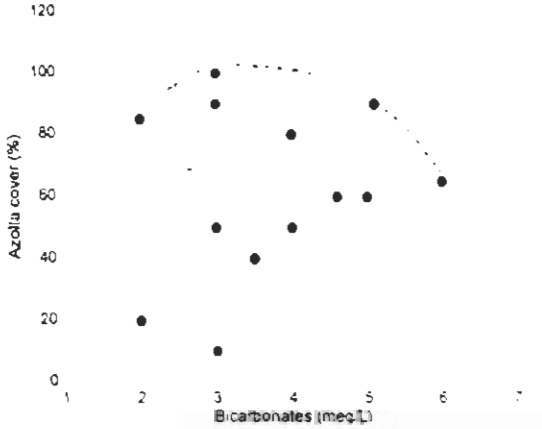

Fig.7e. Effect of floodwater bicarbonates on Azolla cover

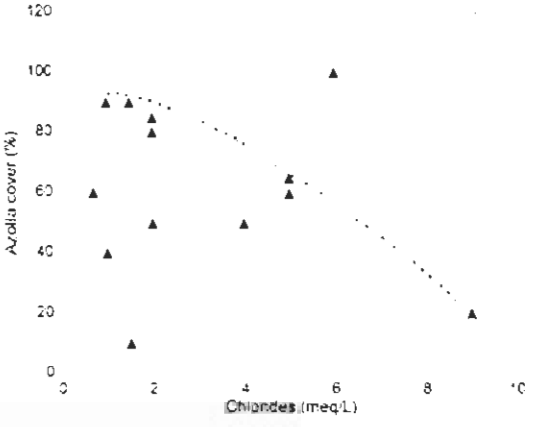

Fig.7f. Effect of floodwater chlorides on Azolla cover

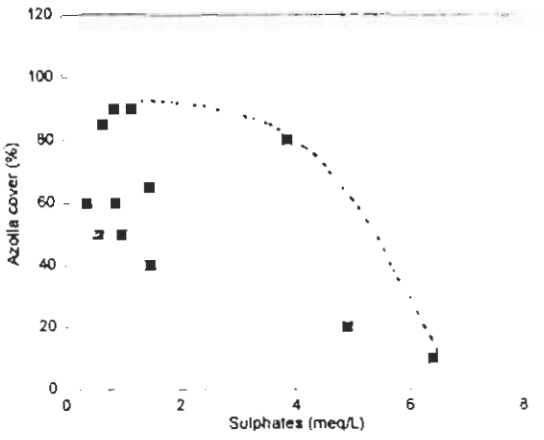

Fig.7g. Effect of floodwater sulphates on Azolla cover.

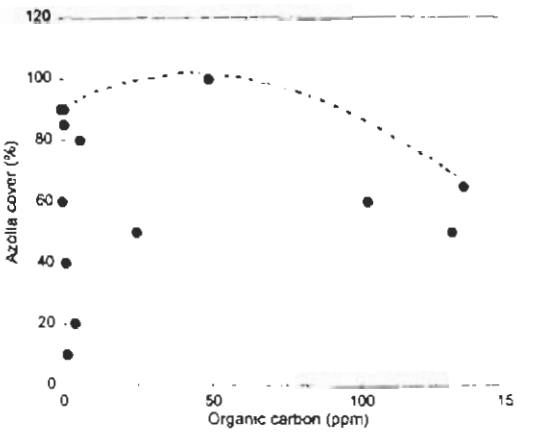

Fig 7h Effect of floodwater organic matter on Azolla cover

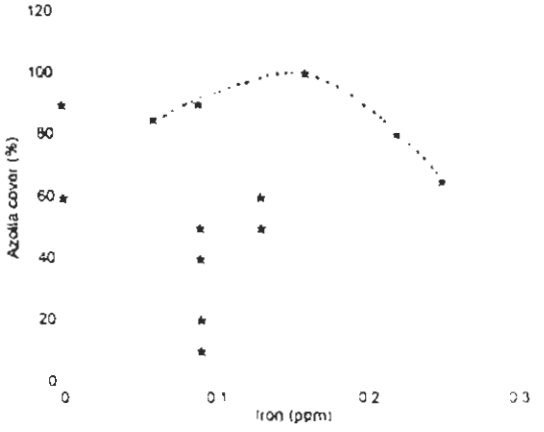

Fig 7i Effect of floodwater iron on Azolla cover

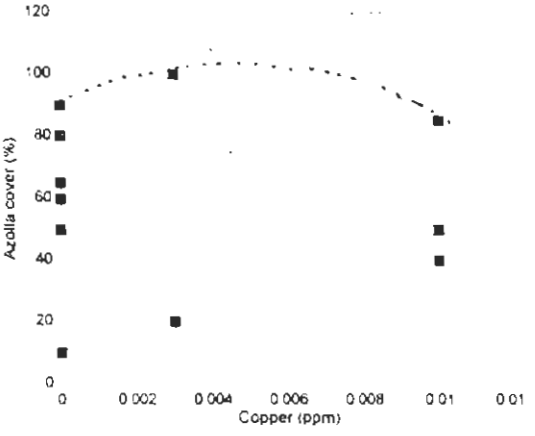

Fig 7j Effect of floodwater copper on Azolla cover

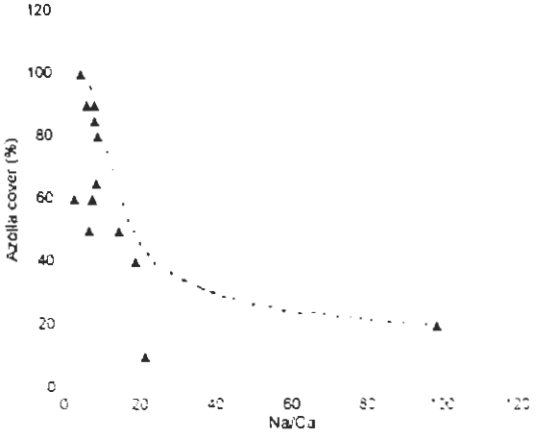

Fig 7k Effect of floodwater Na/Ca on Azolla cover

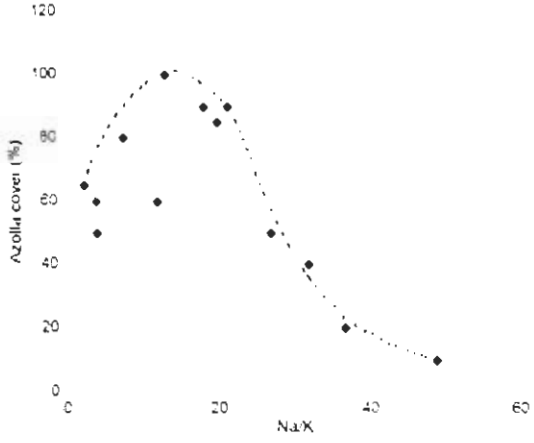

Fig 7l Effect of floodwater Na/K on Azolla cover

May, while only one survived the whole period of severe summer. The heat tolerant strain, which survived at Faisalabad conditions was maintained in the nursery and was used for laboratory, greenhouse and field studies.

On the basis of plant shape, trichomes and shape and size of micro and megasporocarps, the plants were identified as *Azolla pinnata* using the key of Lumpkin and Plucknett (1982). The identification was confirmed by Dr. I. Watanabe, Head Soil Microbiology Department, at IRRI. He, on the basis of detailed study of its morphology and other characters of reproductive structures, identified it as *Azolla pinnata* var. *imbricata*, and is kept in IRRI *Azolla* culture collection under the IRRI code no. PI-0056 NIAB Pakistan (Watanabe et al 1992).

Effect of Temperature on Growth and Sporulation:

Azolla pinnata var. *imbricata* (A.p.i) the local strain, showed varying amount of biomass production at the five different water temperatures and maximum biomass was produced at 30°C (Fig. 8a). The *Azolla* plants showed a decrease in biomass as compared to the inoculated amount of 3g fwt/pot at 36°C, indicating death of plants at higher temperature. In case of fresh culture medium, the increase in fresh biomass (as compared to inoculated amount) was 43, 113, 156, 182% for control (15°C), 20, 25, 30°C respectively, whereas 75% decrease was observed at 36°C.

As compared to fresh culture medium, the effect of water temperature was less prominent for exhausted culture medium as there was no much difference between the amount of biomass produced at different temperatures, however the trend was similar and maximum biomass was also harvested at 30°C than at the other temperatures.

In case of fresh medium, the growth value (amount harvested/amount inoculated) increased almost in a linear fashion at water temperature of 15 to 30°C but it decreased sharply at temperature of 30 to 36°C and was very low (0.25) at 36°C. On contrary to growth value, the doubling time decreased at temperature of 15 to 30°C whereas at 36°C, there was a decrease in biomass as compared to inoculated amount. There was a sharp decrease in doubling time at temperature of 15 to 20°C and then the decrease was relatively gradual upto 30°C and it was negative at 36°C, indicating more effect of increase in temperature from 15 to 20° and from 30 to 35°C (Fig. 8b).

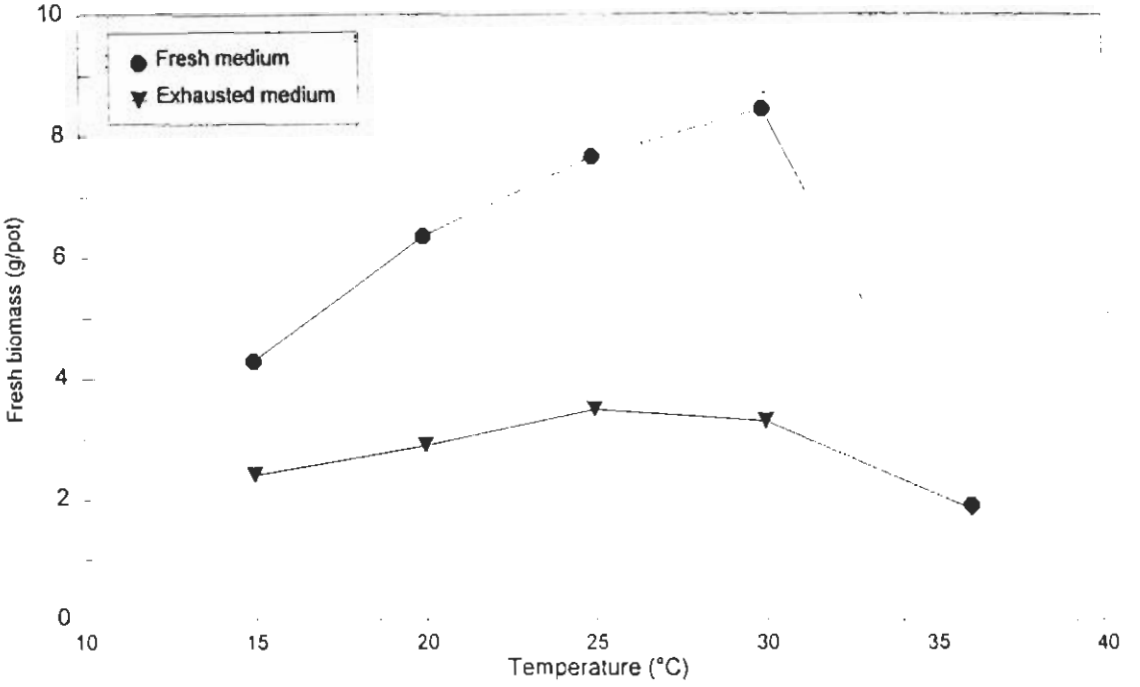

Fig. 8a. Effect of water temperature on Azolla growth.

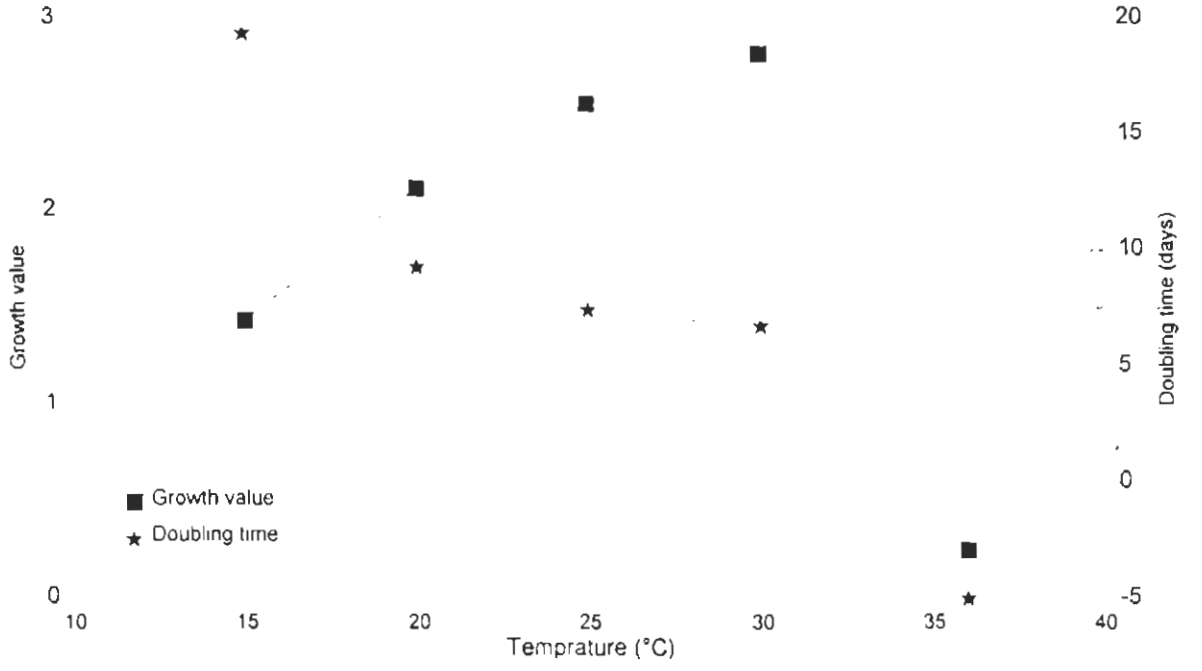

Fig. 8b. Effect of water temperature on growth value and doubling time of Azolla.

The morphological observations made during this experiment showed that *Azolla* plants were green to reddish green, of larger size and healthy at 25 and 30°C, in case of fresh medium as well as in exhausted medium. The plants were more reddish in colour at 20°C and 36°C, but they were of medium size at 20°C while of much smaller size with brownish older parts at 36°C.

After about a week or so the plants growing at water temperature of 25 and 30°C developed both micro and megasporocarps in pairs whereas no reproductive structures were found in case of plant growing at 15, 20 and 36°C. The microsporocarps were yellowish in colour and globular in shape and microsporangia were also visible through the thin covering of microsporocarps. The microsporocarps were 1.5 mm long, 1.4-1.5 mm in diameter, and had a conical indusium at the tip. The megasporocarps were smaller in size being 1mm long and 0.5 mm in diameter having brownish-black indusium at the tip, central white body and a brownish-black basal part.

AZOLLA CULTIVATION

Azolla Cultivation in Defined Culture Media:

Biomass, Chlorophyll Content and ARA: The comparison of *Azolla* growth in 5 different culture media comprising 4 culture media reconstituted according to floodwater analysis (where *Azolla* growth was better) and *Azolla* medium of IRRI, indicated that maximum dry biomass/pot in 35 days from an inoculum of 1.0g fresh *Azolla* (0.2g dwt), was produced in IRRI medium having pH 6.5. When pH of IRRI medium was adjusted to 8.0, which is the common pH of floodwater of rice areas of Punjab, a higher biomass was produced in KB medium followed by IRRI, KA, MD and BT culture media (Fig. 9a). The growth value, computed on dry weight basis, was 34, 19, 14, 9, 8.7 and 7.6 g/pot for IRRI medium pH 6.5, KB, IRRI medium pH 8.0, KA, MD and BT culture medium respectively. When the pH of all the culture media was adjusted to 8.0, KB medium produced 36% more *Azolla* biomass than IRRI medium, and more than double biomass was obtained as compared to that from other 3 culture media tested in this experiment.

Like dry biomass production, the chlorophyll content, in *Azolla*, showed similar pattern (Fig. 9a), however in MD medium, it was slightly higher than that in KA medium.

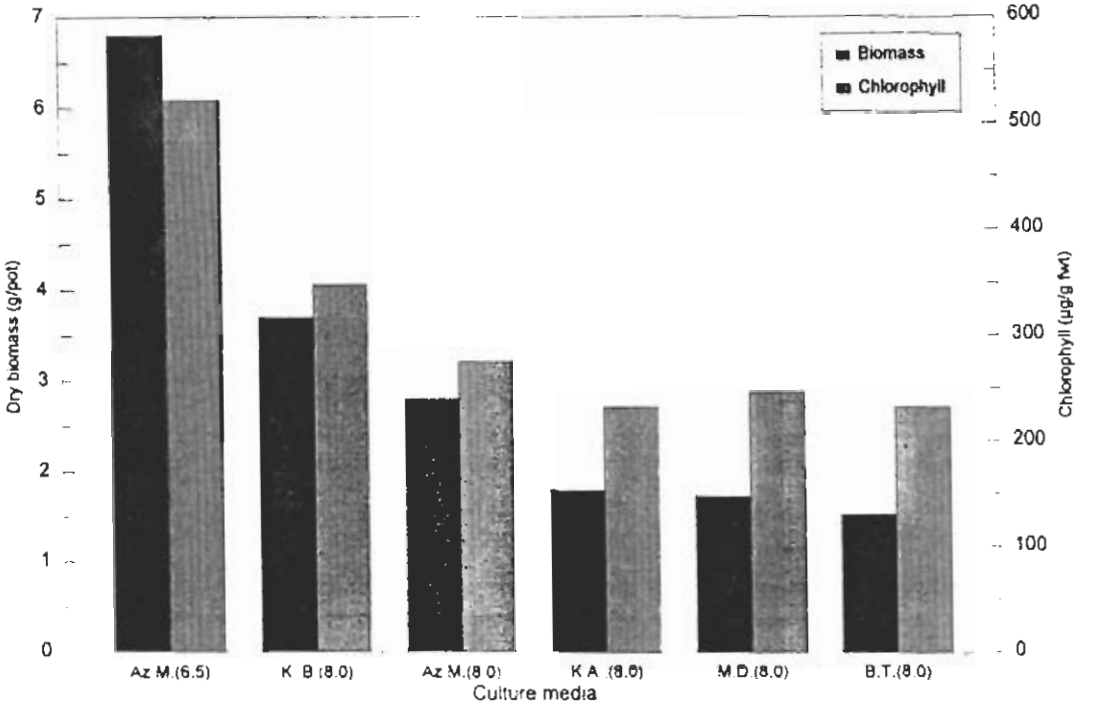

Fig. 9a. Azolla growth in different culture media at pH 8.0 (Azolla Medium pH 6.5 & 8.0)

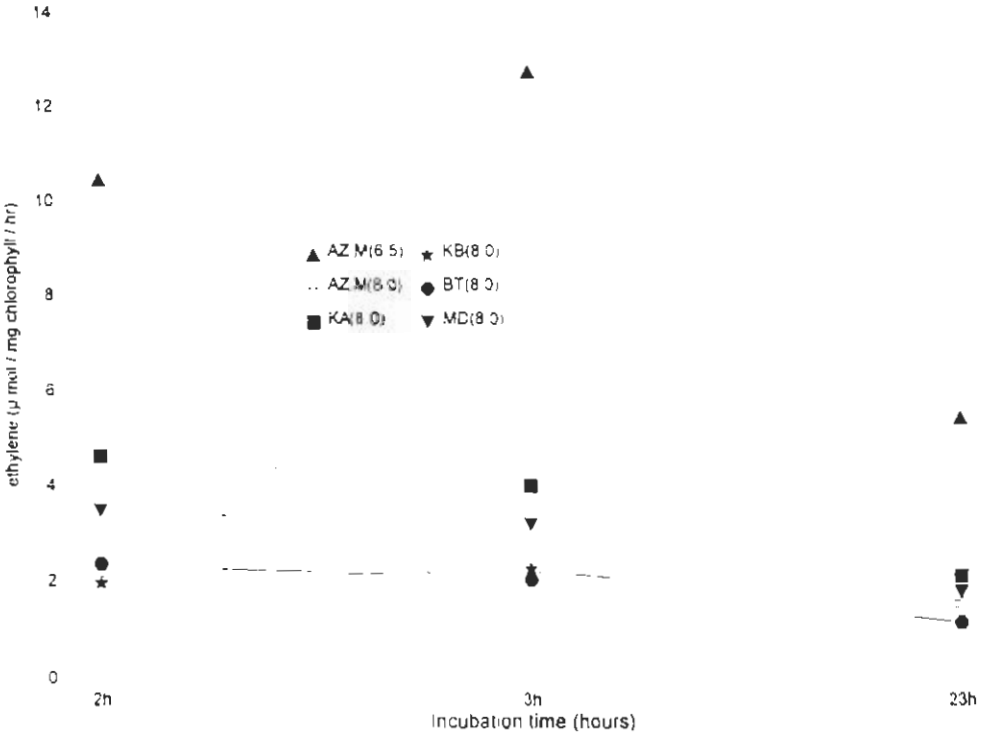

Fig. 9b. Nitrogenase activity after different incubation times in different culture media.

The chlorophyll content was 522, 348, 275, 232, 246, and 232 for IRRI (pH 6.5), KB, IRRI (pH 8.0), KA, MD and BT culture medium respectively.

The nitrogenase activity as measured by ARA, indicated that it was much higher in IRRI medium (pH 6.5), than that in the IRRI medium (pH 8.0) and other culture media also adjusted to pH 8.0 (Fig. 9b). However, when all the culture media were set at pH 8.0, the amount of nitrogenase activity using ARA in KA medium was higher than that obtained from all other culture media. At pH 8, the ethylene production for 2-hour incubation were, 4.7, 3.5, 2.4, 2.3 and 2.1 μmol/mg chlorophyll/hr from KA, MD, BT, IRRI and KA culture medium respectively. In general, the rate of reduction of acetylene to ethylene by nitrogenase enzyme, was higher for 2 hour incubation than that for 3 hour or 23 hour incubation, except for IRRI medium in which it was higher at 3 hour incubation period, and relatively more decline in values from ARA was seen at 23 hour incubation period.

Effect of Humic Acid on Growth and N_2 Fixation: The effect of different concentrations on humic acid (HA) in N-free KB medium indicated that *Azolla* growth increased with increase in HA concentrations used in this experiment (Fig. 10a). The increase in growth value was more prominent for increase in HA from 0.0175 to 0.035%, while a lesser effect was observed for further increase in HA concentration. The growth value was 12.4, 14.7, 19.3 and 20.9 with 0, 0.0175, 0.035 and 0.052% HA, indicating that about 1.5 time increase in biomass production can be achieved by using a small amount (0.035%) of HA in the culture medium.

The reduction in doubling time were linear upto concentration of 0.035% HA, and further increase in HA concentration was not so effective (Fig. 10a).

The effect of HA addition on nitrogenase activity in *Azolla* as indicated by reduction of acetylene to ethylene, was positive (Fig. 10b). The increase in ethylene production rate increased linearly with an increase in HA concentration, and unlike growth value and doubling time, for all the HA concentrations, used in this experiment, increased the nitrogenase activity increased progressively.

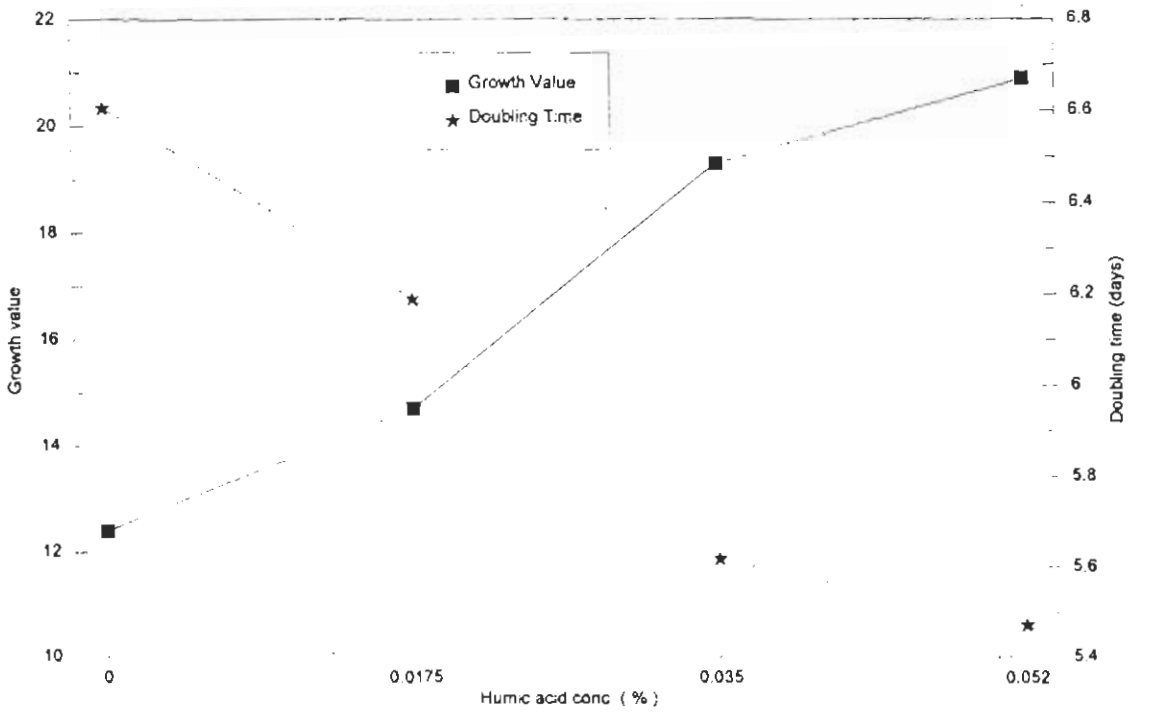

Fig. 10a. Effect of humic acid on growth value and doubling time of Azolla, in KB medium.

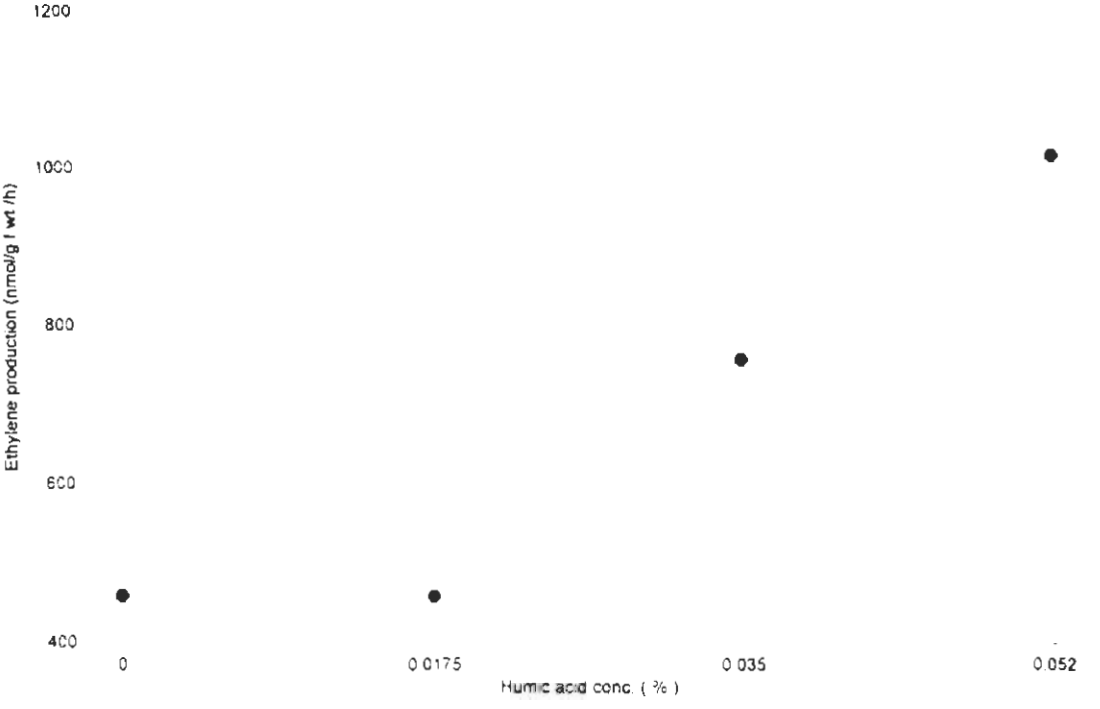

Fig. 10b. Effect of humic acid on nitrogenase activity of Azolla, grown in KB medium

Azolla Cultivation in Undefined Culture Media:

The results of preliminary experiment using different soils and waters, and comparison of defined and undefined culture media for *Azolla* growth and nitrogenase activity, and long-period growth in soil-water culture are being given below.

Preliminary Experiment: The preliminary experiment to know the effect of different soils and waters on *Azolla* growth indicated that local pond soil+ pond water+ cow dung was better than the original Gujranwala soil+local water, local canal water+Fe+Mn and N-free BG11 culture medium (Fig. 11), as number of *Azolla* plants/pot after 4 weeks of incubation was 62, 53, 45 and 34 respectively.

Comparison of Defined and Undefined Culture Media: The comparison of defined and undefined culture media for supporting *Azolla* growth for 4 months indicated that soil-water culture medium having 5% soil+0.5% FYM was better than the remaining defined and undefined culture media (Fig. 12a). The fresh biomass produced was 470, 565, 594 and 660g per pot for KB, IRRI, 0.5% FYM and 5% soil+ 0.5% FYM culture medium respectively. Although minimum doubling time (12.8 days) was observed for soil+FYM culture, yet there was not much difference in doubling time between different culture media and it ranged from 12.8 to 13.5 days in these 4 culture media.

The biomass production in KB, 5% FYM and 5% soil+0.5% FYM culture medium indicated that fresh biomass produced in 24 days was 6.2, 10.4 and 13.4g per pot, respectively (Fig. 12b). The doubling time was 6.6, 5.5 and 5.1 days for KB, FYM and soil+FYM culture medium respectively.

The ethylene production by the nitrogenase enzyme also followed the pattern of biomass production but the difference was more pronounced between FYM and soil+FYM culture medium (Fig. 12b). The rate of ethylene production was 460, 710 and 1420 nmol/g fwt/hr, for control (KB medium), 0.5% FYM and 5% soil+0.5% FYM respectively, indicating about double rate of nitrogen fixation in soil+FYM than that in only FYM or KB medium.

Long Period Cultivation in Soil-Water Culture: For maintaining *Azolla* nursery in greenhouse conditions, the soil-canal water culture maximum amended with different nutrients indicated that maximum fresh *Azolla* biomass was produced in soil-water culture

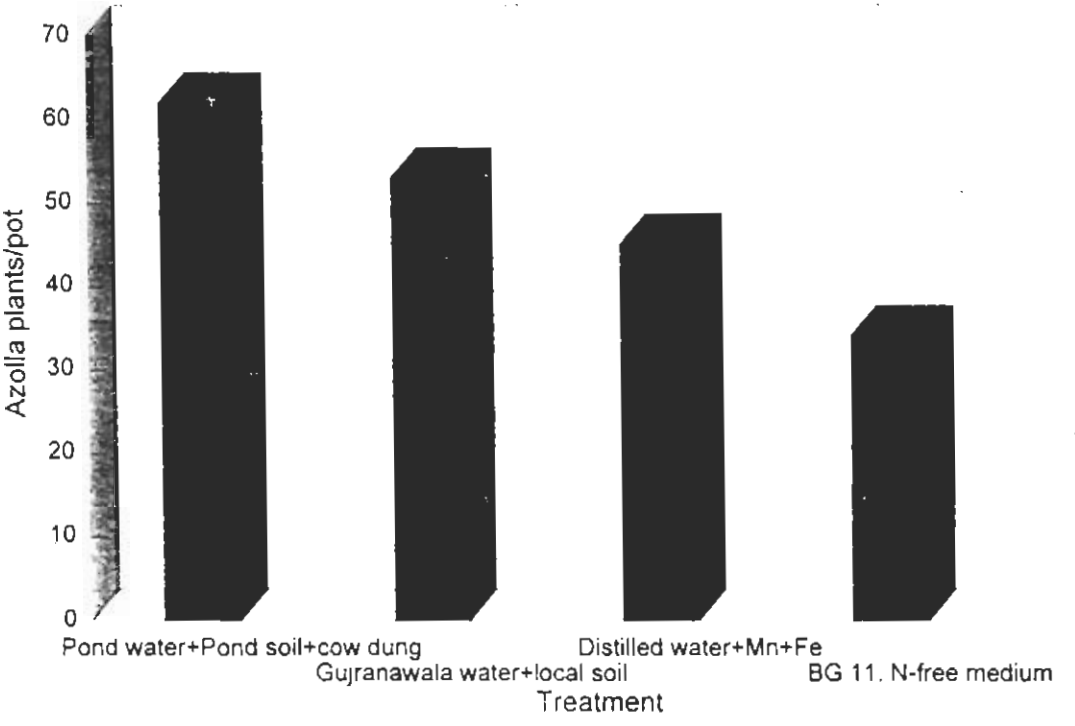

Fig. 11. Effect of type of soil and water and nutrients on Azolla growth.

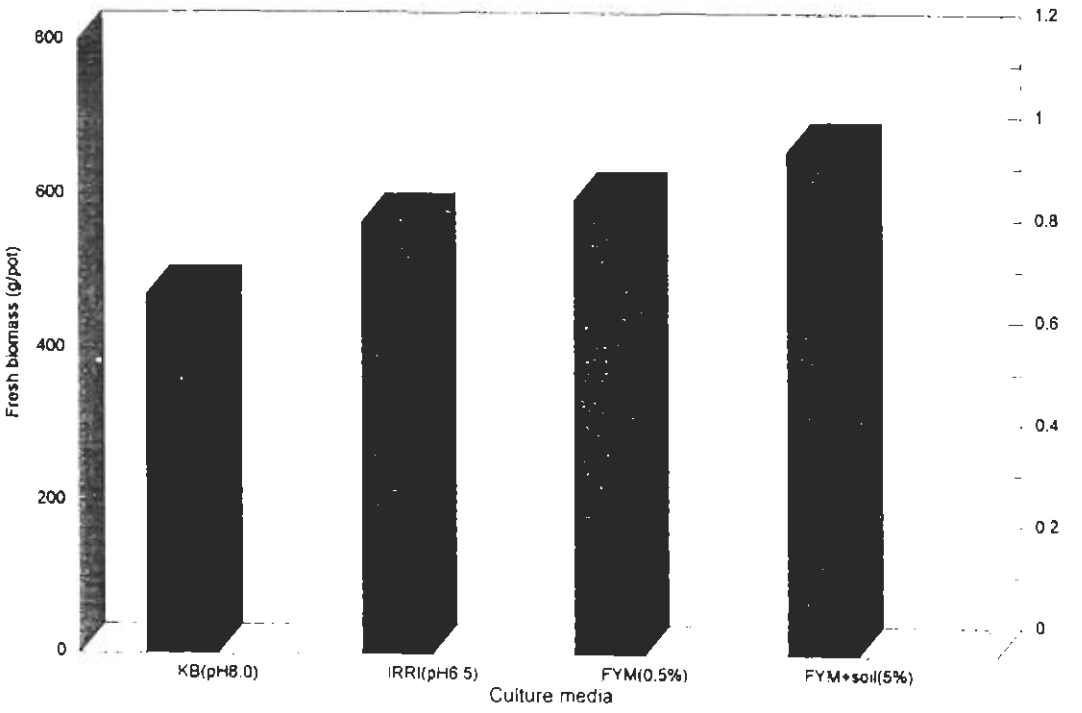

Fig. 12a. Comparison of Azolla growth in defined and undefined culture media.

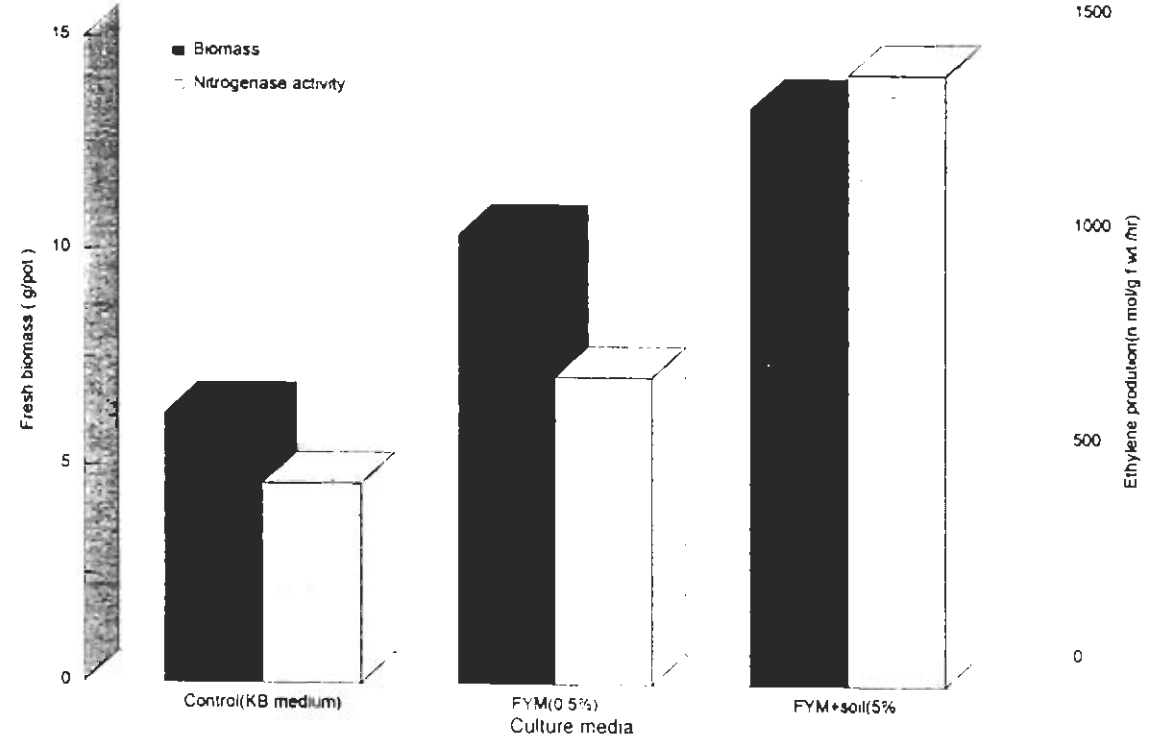

Fig. 12b. Effect of farmyard manure on Azolla growth and nitrogenase activity.

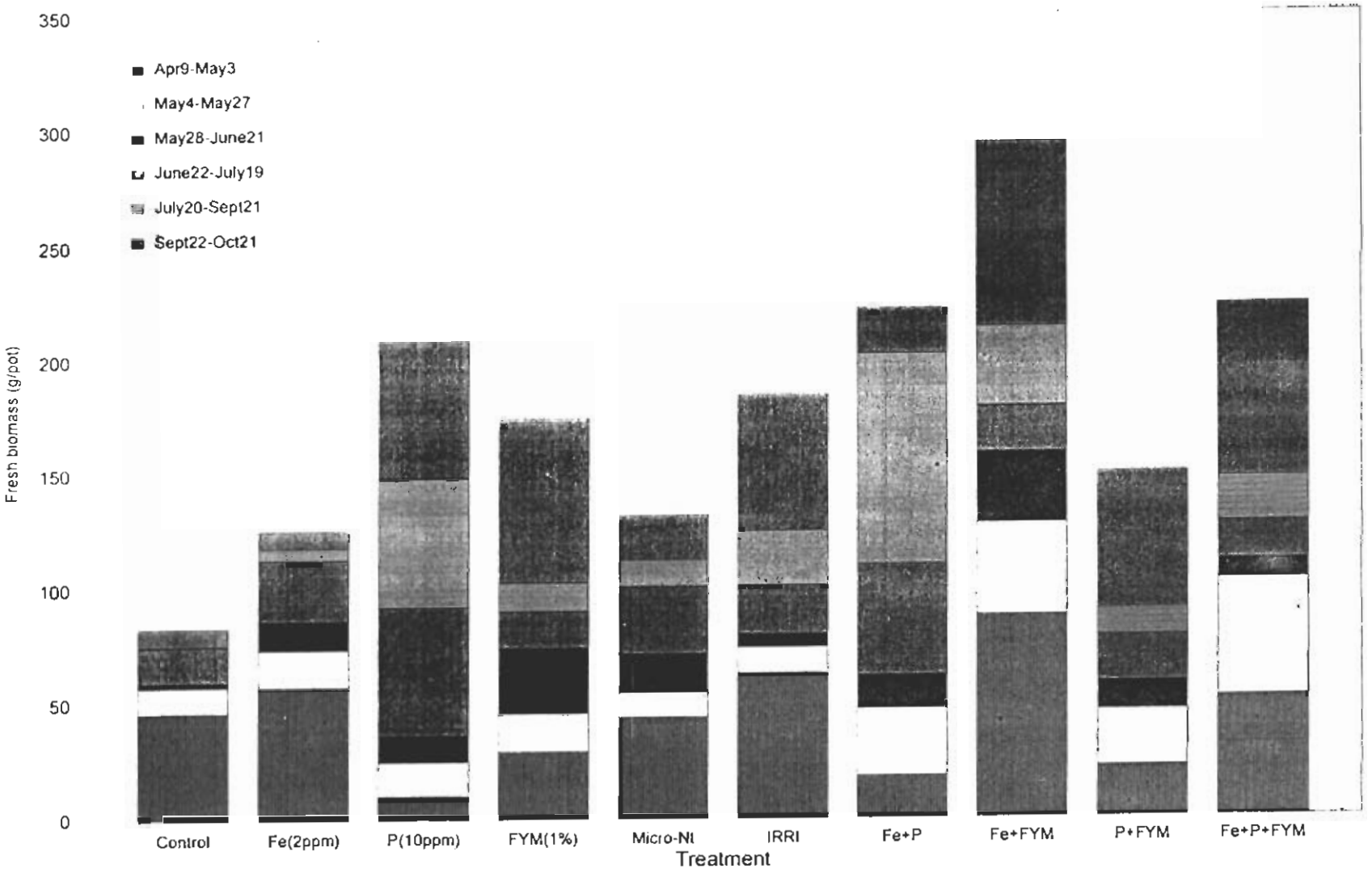

Fig. 13. Effect of nutrient addition to soil-water culture on cumulative Azolla biomass production.

medium having 2ppm Fe+1% FYM during a period of about 7 months i.e. April 9 to Oct 21 (Fig. 13). The cumulative *Azolla* biomass produced for the 10 treatments in increasing order was: Control 23, <Fe 125, <Micronutrient 132, <P+FYM 150, <FYM 175, <IRRI Medium 185, <P 209, <Fe+P 222, <Fe+P+FYM 224, <FYM+Fe 294 g/pot.

In the beginning period (April 9-May 3), the addition of P either alone or with iron or with only FYM, depressed *Azolla* growth, while Fe alone or in combination with FYM improved its biomass production. During the hottest and dry period of May 28-June 21 (Fig. 5) a higher amount of *Azolla* biomass was produced in FYM and Fe+FYM amended culture solutions, while a higher growth of *Azolla* was found in P and Fe+P during hotter but lesser dry period of June 22 to July 19. The biomass produced by micro-nutrient was slightly higher than Fe application (Fig. 13).

The morphological observation made during the above said periods (on May 20, June 6, 14, 21, July 12, 19, and Aug 2) indicated that the plants were mostly yellowish-green, smaller in size, having slender roots with many root hair in pots of control, Fe, and micronutrient, whereas green to dark green, medium to large in size, with thicker roots in pots having Fe+P, FYM, FYM+Fe, FYM+P, and IRRI medium. The observation, made in the next year during the 3rd week of January i.e. after about 10 months of start of the experiment, showed that on the basis of number of plants per pot and healthiness of plants, the best treatments were FYM+Fe, FYM+Fe+P and FYM, and better ones were P, IRRI medium, and FYM+P, while the remaining treatments i.e. micronutrients, Fe+P, Fe and control were poor ones.

Diagnosis of Nutritional Constraints: The nutrition missing technique used for diagnosis of P, Zn and Fe in the paddy soil indicated that P was the major limiting nutrient for *Azolla* growth in the tested soil (Fig. 14a). The dry biomass produced in 4 weeks was 485, 521, 978, 805, and 1024 mg/pot for Control(-P-Zn-Fe), -P(+Fe+Zn) -Zn(+P+Fe), -Fe(+P+Zn) and having all the 3 nutrients (+P+Zn+Fe) respectively. As compared to all + nutrient treatment (+P+Zn+Fe) the lowest biomass produced in -P than the other 3 treatments indicated that P was the major limiting nutrient in this soil for *Azolla* growth. The next limiting nutrient was Fe, while Zn was not problem as in -Zn there was not much reduction in biomass production as compared to all + (+P+Zn+Fe) treatment. The

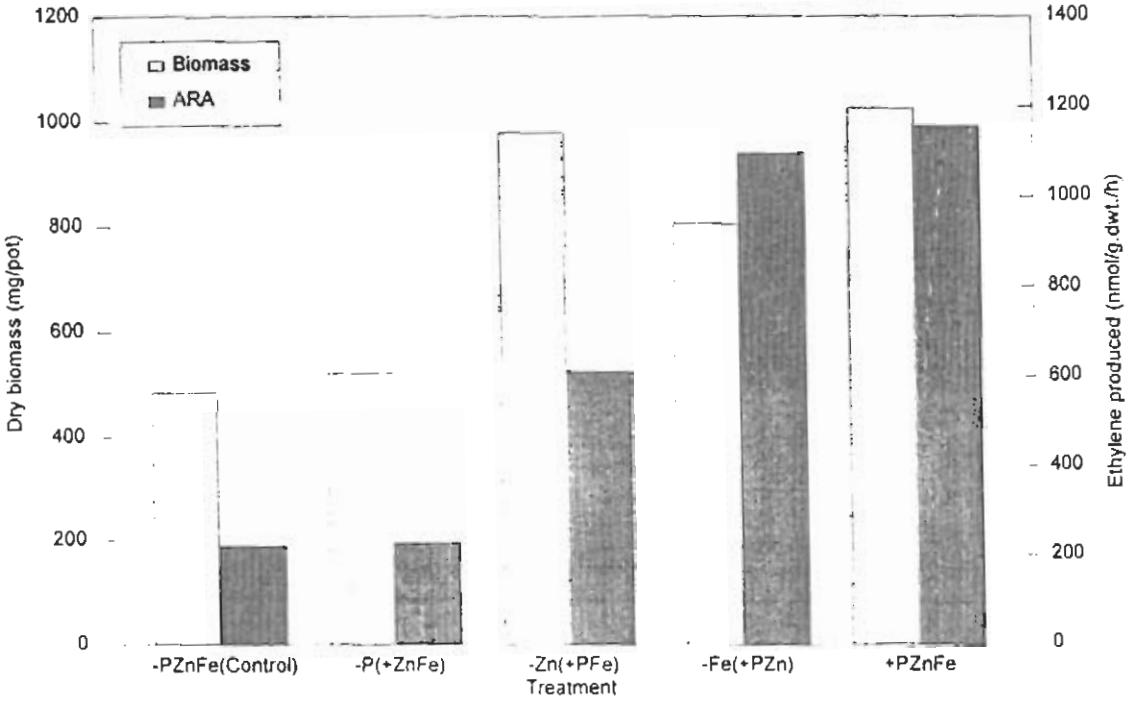

Fig. 14a. Diagnosis of nutritional constraints for Azolla growth in a paddy soil.

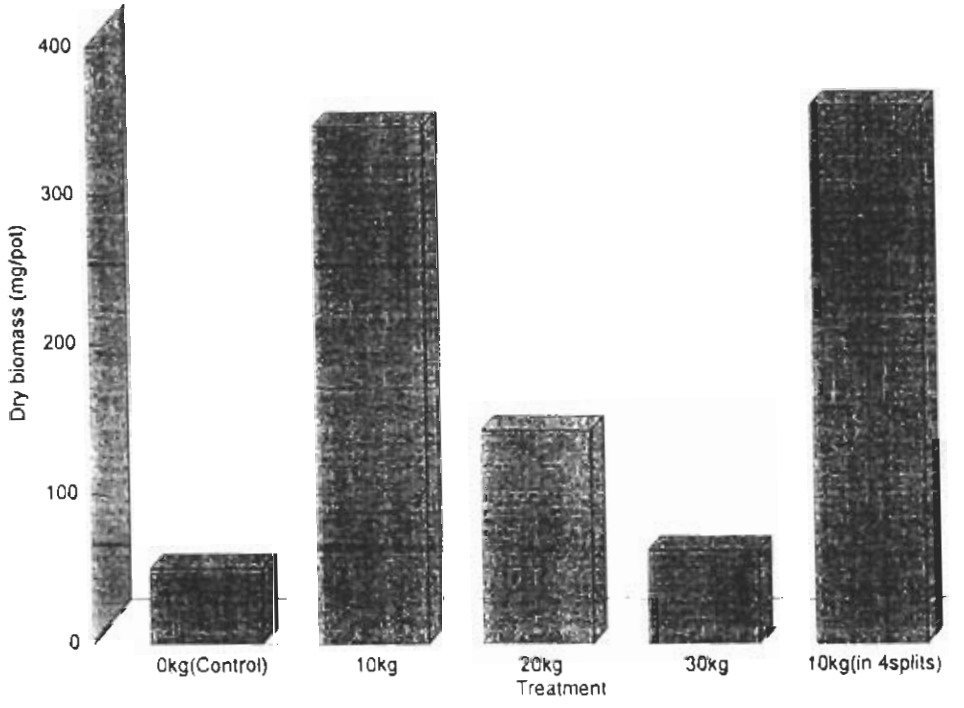

Fig. 14 b. Effect of different doses of P on Azolla growth in the paddy soil.

morphology of *Azolla* also indicated smaller and reddish weak plants in -P and somewhat yellowish and chlorotic plants in -Fe pots.

The nitrogenase activity, as indicated by ARA, also indicated a general pattern similar to biomass production, however less activity was observed for -Zn than -Fe treatment (Fig. 14a).

On the basis of above experiment, the next experiment designed to find the optimum dose of P, indicated that a lower dose of P was better than the higher ones (Fig. 14b). The biomass produced was 50, 346, 143, 62 and 361 mg/pot for 0 (Control), 10, 20, 30kg P_2O_5/ha applied once, and 10 Kg P_2O_5/ha (in 4 splits) treatment respectively. The 10 kg P_2O_5/ha in 4 splits (2.5 kg/week) was slightly higher (4%) than the application of same amount of P fertilizer at one time.

PEST MANAGEMENT

Pests and their Control:

During cultivation of *Azolla* in greenhouse and field nursery, some pest were found feeding on *Azolla*. Water snails belonging to genus *Lymnaea* family Lymnaeidae, subclass pulmonata and class Gastropoda (one piece shell) used to eat *Azolla* roots and tender growing tips of *Azolla* rhizome throughout the year. Similarly, discoid snails of genus *Gyraulus* family Planorbidae of class Gastropoda ate up *Azolla* plants. In addition to these larger pests, very small bivalve crustaceans belonging to class Ostracada used to eat *Azolla* roots and hence retard its growth and they were more active in summer.

The attack of *Nymphula* sp. (Lepidoptera) was seasonal, being more active in February to March and in September to October. The larvae wrapped up the *Azolla* plants around their bodies, and hid inside the tunnel made from *Azolla* frond. They were very efficient in eating *Azolla* as they ate up a lot of Azolla plants in a very short period of time. Sometimes fungus was found growing on *Azolla*, but its growth was observed only on the dead plants damaged by insects and thus as such the fungus was not a problem in *Azolla* cultivation.

To find a suitable pesticide and its minimum concentration to kill *Azolla* pests, water snails of *Lymnaea* sp. were used. The short term exposure of different insecticides showed

that very low concentration (5 ppm a.i.) of Endrin (Chlorinated hydrocarbon) and Akar (Chlorobenzilate emulsion) while a relatively higher concentration (30 ppm a.i.) of Furadan (Carbofuran), Heptachlor (Chlorinated hydrocarbon) and Nuvacron (Monocrotophos) were required to kill 90% of the snails (Fig. 15a).

Effect of Pesticides on *Azolla* Growth:

On the basis of above results, the minimum concentration of lethal dose killing 90% (LD 90) of the snails, tested on *Azolla*, indicated that for Azolla growth the toxicity order (low to high) was Furadan < Nuvacron < Heptachlor < Akar < Endrin (Fig. 15b). The *Azolla* biomass harvested after 3 weeks of cultivation was 5.5, 5.0, 4.0, 3.2, 0.8 and 0.1 g/pot for control (soil-water culture medium without any insecticide), Furadan, Nuvacron, Heptachlor, Akar and Endrin respectively. Furadan treated pots gave 91% of the biomass compared with that obtained from of control, while Nuvacron 73%, Heptachlor 58%, Akar 15% and Endrin only 2% of control. Thus Furadan, which was least harmful to *Azolla*, was selected as pesticide for *Azolla* nursery. It was also found that Furadan was capable of controlling other pests like discoid snails, Ostreads and *Nymphula* larvae.

NITROGEN FIXATION IN *AZOLLA*

The results of greenhouse experiment on nitrogen fixation in the presence of combined N, and nitrogen fixation estimated in the field condition using ^{15}N dilution technique, are given below.

Nitrogen Fixation in the Presence of Combined N:

The effect of combined N on *Azolla* biomass production and nitrogen fixation as estimated by ARA in the greenhouse study indicated that combined-N affected both of these processes (Fig. 16a,b). The biomass increase over inoculum as indicated by growth value, showed that addition of a small amount of ammonium N (14 ppm) increased fresh biomass production in *Azolla* to about 1.5 times of N-free KB medium used as control. However, further increase, in combined N at the rate of 28 and 42 ppm, caused a decrease in growth value, but it was still slightly higher than N-free control (Fig. 16a).

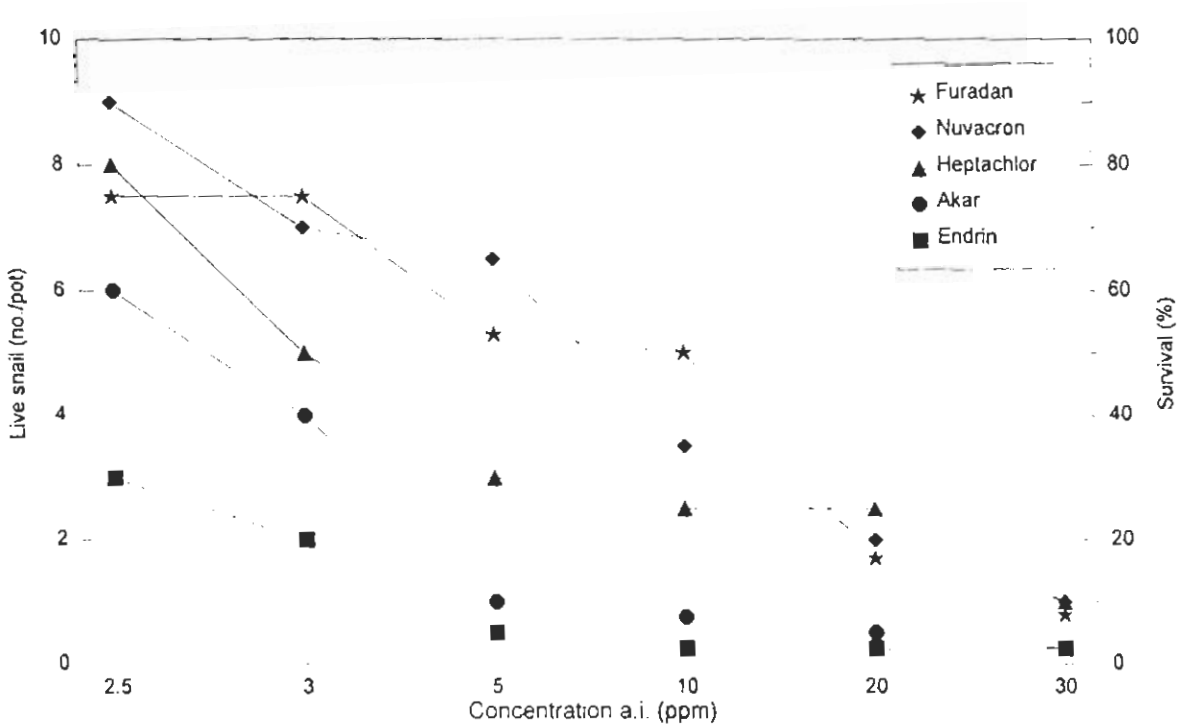

Fig. 15a.Effect of different concentrations of pesticides on survival of snails.

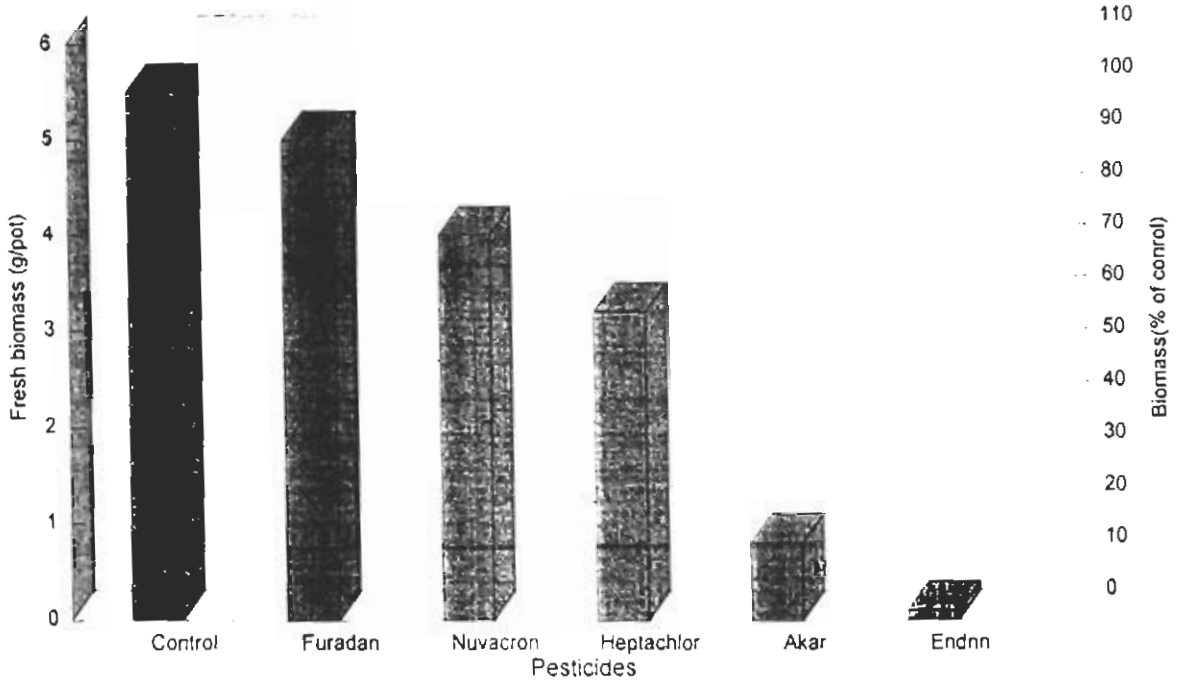

Fig. 15b.Effect of snail-lethal concentration (LD 90) of pesticides on Azolla growth.
(LD 90 = 5ppm for Aakar & Endrin while 30ppm for others)

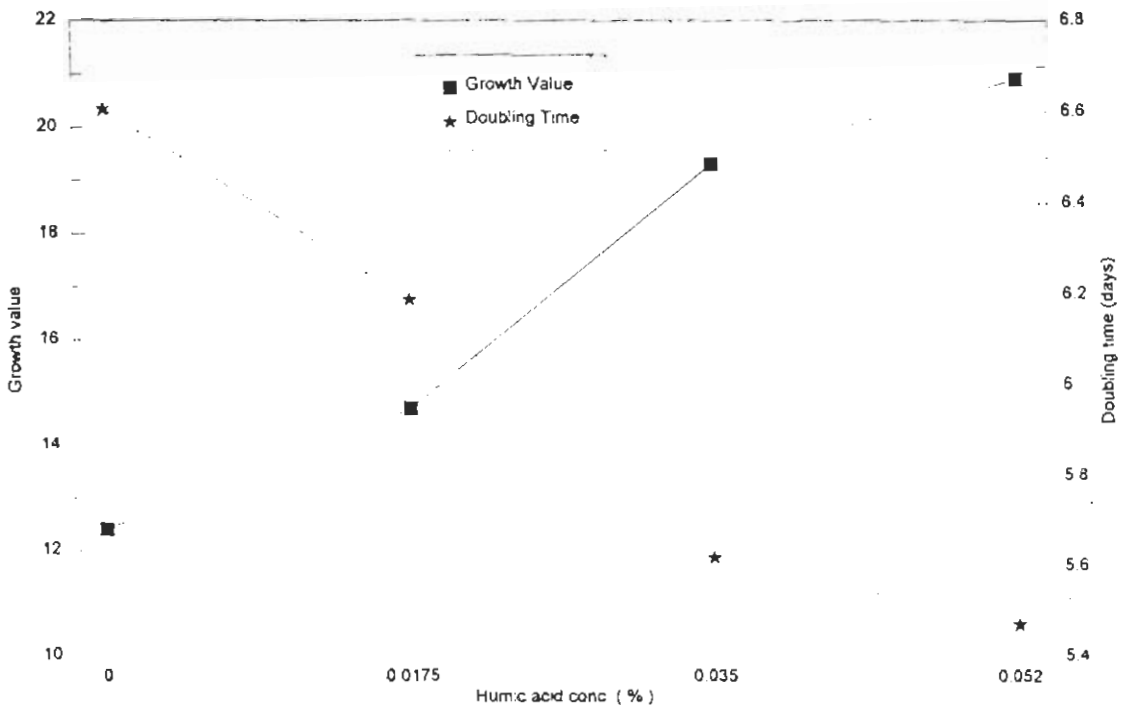

Fig. 16a. Effect of humic acid on growth value and doubling time of Azolla, in KB medium.

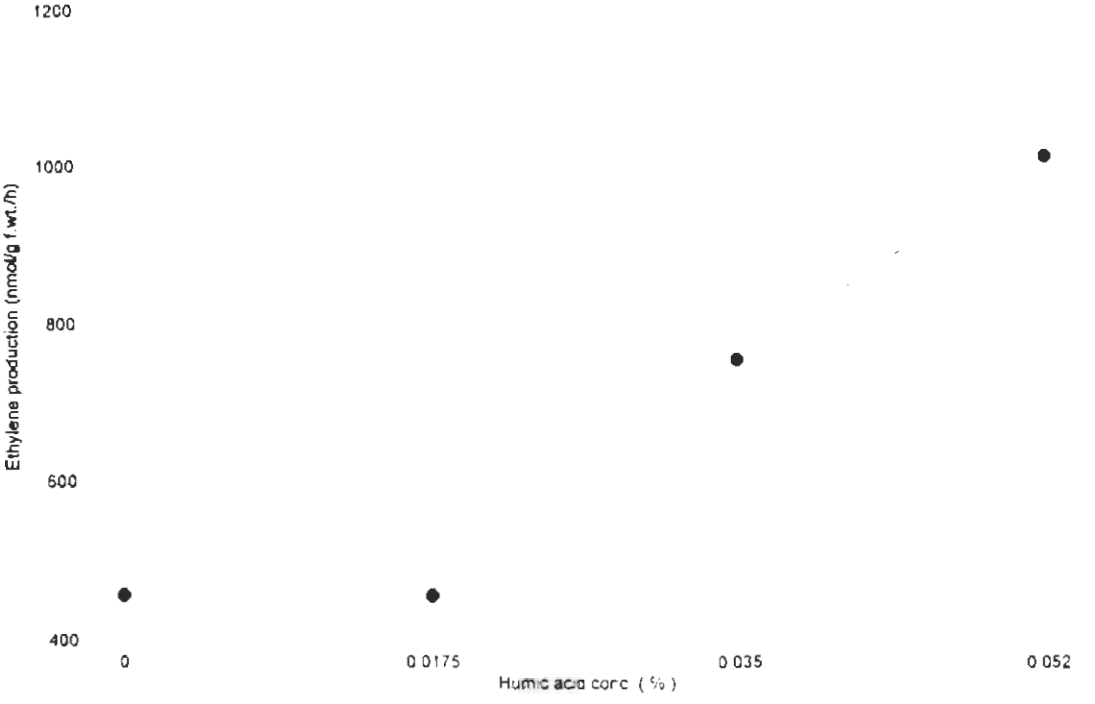

Fig. 16b. Effect of humic acid on nitrogenase activity of Azolla, grown in KB medium.

The doubling time sharply decreased from 6.6 to 5.8 days with the lowest dose (14 ppm) of combined N but it increased with further increase in ammonium-N concentration in the medium, however it was slightly lower than of control (Fig. 16a).

The effect of combined N on the nitrogenase activity was depressive (Fig. 16b). The amount of ethylene produced/g fwt/hr decreased by 18% as compared to control with the lowest dose of combined N (14 ppm), and further increase in the concentration of ammonium-N (28 and 42 ppm) also decreased the nitrogenase activity but with a lesser decline in its activity as compared to decrease from control to 14 ppm-N concentration.

Estimation of N_2 Fixation by ^{15}N Dilution Technique:

The nitrogen fixation by *Azolla*, computed after growing it in ^{15}N enriched soil for 3 weeks in the field conditions and using *Lemna minor* as the reference plant, indicated that major amount of N in *Azolla* was derived from the air (Fig. 17a). The amount of N accumulated through nitrogen fixation from air was 68, 75, 80, 81 and 83% for *A. pinnata* var. *imbricata* (local strain), *A. caroliniana*-301, *A. caroliniana*, Rong-ping (hybrid *Azolla*) and *A. microphylla*-418 respectively.

The rice grown intermixed with *Azolla* in the same sieve (harvested after 3 weeks) had lower ^{15}N abundance than the rice grown without *Azolla* in separate sieves. The nitrogen derived from air (NdfA) was computed (using the rice without *Azolla*, as reference plant) and it was found that 21% of N in rice was derived from air when grown intermixed with *Azolla*. Secondly the rice dry biomass produced, when grown with *Azolla*, was about 2.5 times higher as compared to rice biomass produced without *Azolla* (Fig. 17b).

CARBON AND NITROGEN MINERALIZATION

Decomposition of *Azolla* in Soil:

Decomposition of Unlabelled *Azolla*: The mineralization of *Azolla*-carbon from unlabelled *Azolla* incubated at 30% and 100% water saturation percentage of the soil for 40 days, at 25-32°C in the laboratory conditions indicated that decomposition rate was faster during the first 10 days and this process was less active in the later incubation period (Fig. 18a). The rate of Azolla-C mineralization as indicated by CO_2 evolution indicated that it was

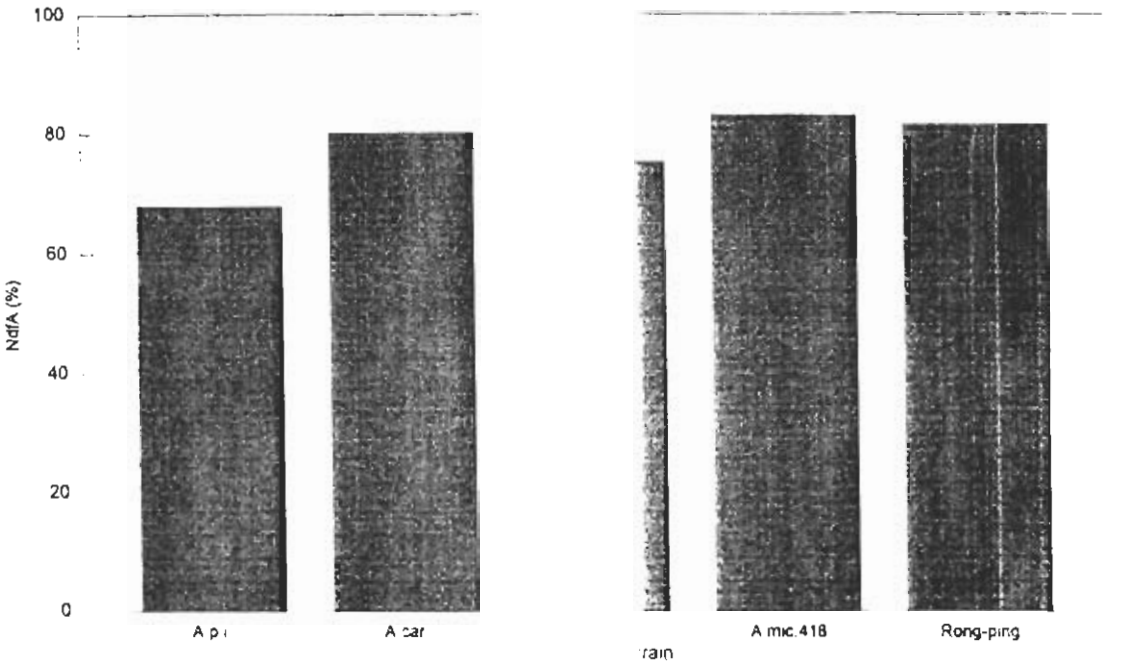

Fig. 17a. Percent nitrogen derived from at
(Using N-15 dilution techni
dfA) in Azolla,grown in field conditions.
inor as reference plant)

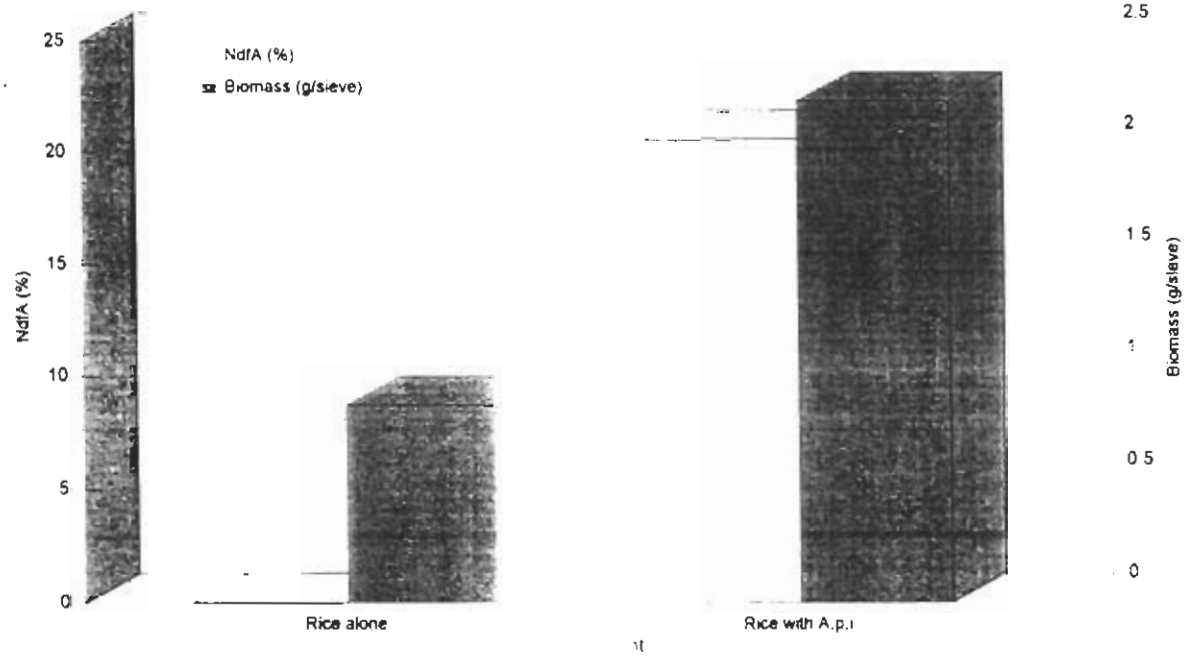

Fig. 17b. Positive effect of Azolla on rice growt
(Using N-15 dilution technique an
rice. grown intermixed in one sieve
ce. as reference plant)

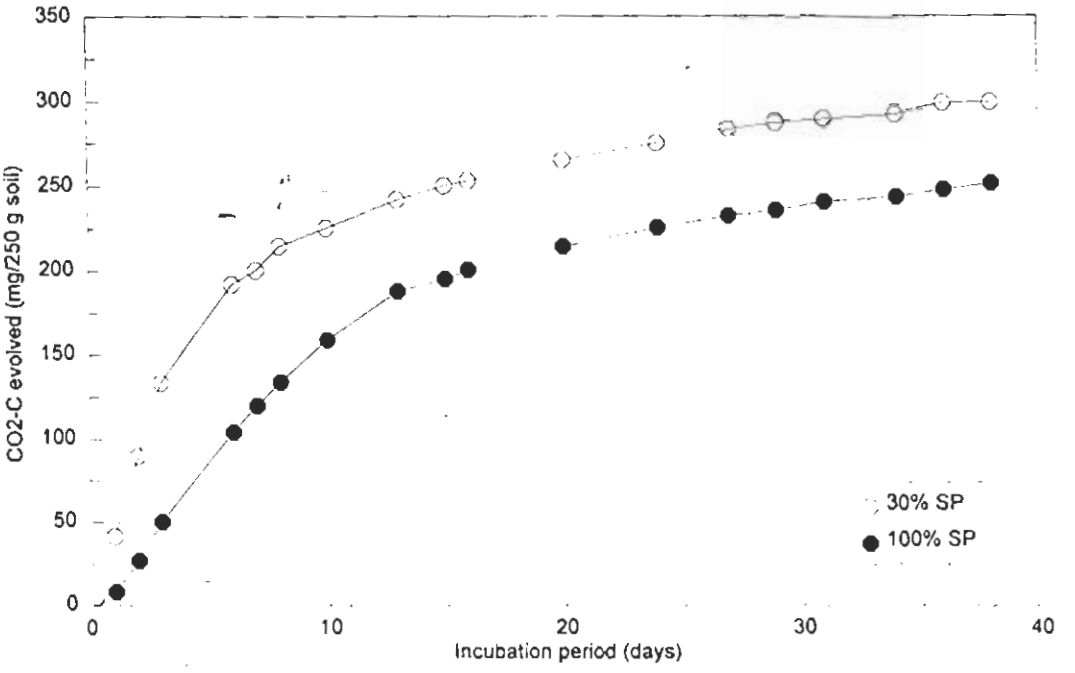

Fig. 18a. Decomposition of unlabelled Azolla in soil at two moisture levels.
(SP = Saturation precentage of soil)

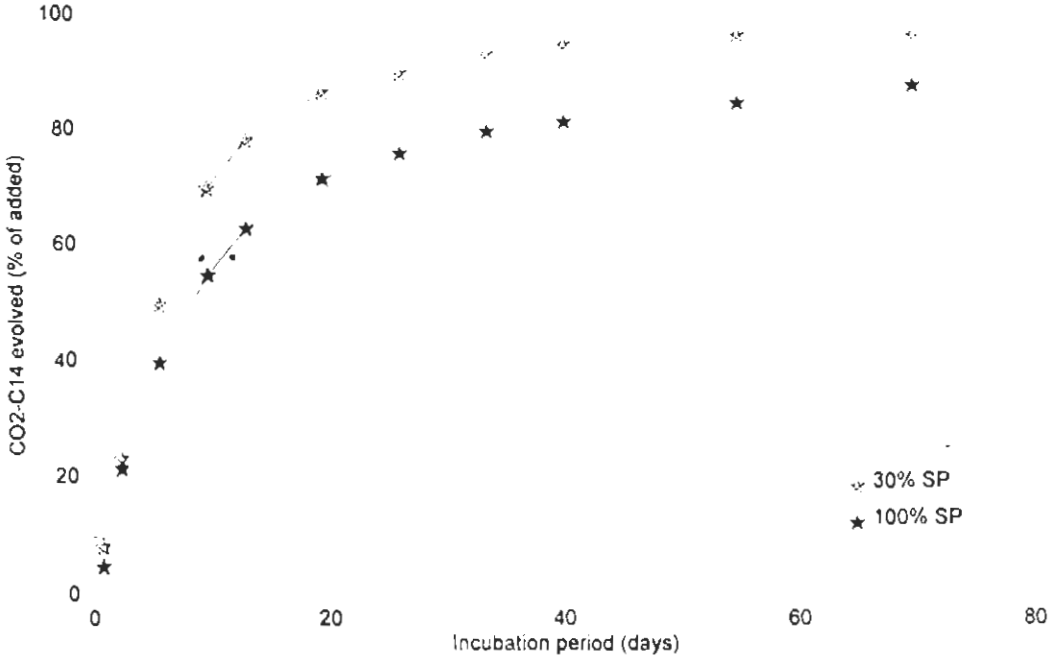

Fig. 18b. Decomposition of C-14 labelled Azolla in soil at two moisture levels.
(SP = Saturation precentage of soil)

at higher rate for 30% SP, than 100 SP throughout the incubation period. In case of 30% SP the cumulative CO_2 evolution increased from 41 mg/250 g soil (164 mg/kg) to 200 mg/250 g soil by 7th day, reached to 250 mg by 15th day and to 299 mg/250 g soil by 38th day of incubation. The decomposition of *Azolla* at 100% SP was slower than that at 30% SP (Fig. 18a) as it increased from 8 mg/250 g soil to 120 mg by 7th day, 195 mg by 15th day and 251 mg/250 g soil by 38th day of incubation.

Decomposition of ^{14}C-Labelled *Azolla*: The *Azolla*-C mineralization from the ^{14}C-labelled *Azolla* also showed similar pattern of ^{14}C losses through CO_2-^{14}C evolution for 30% and 100% SP soil moisture level for the 80 day incubation period in laboratory conditions (Fig. 18b). Here, like above experiment, mineralization was faster in soil at lower moisture level than in the water-saturated soil. In case of 30% SP, 50% of ^{14}C from *Azolla* was evolved as CO_2 by the end of first week, while about 78% ^{14}C by the end of second week, 90% by the end of 4th week, 95% by the end of 6th week and 97% by the end of 10th week of incubation. The rate of mineralization was relatively slower in 100% SP, as 40% of added ^{14}C was evolved by 7th day, 63% by 14th day, 77% by 28th day, 83% by 42th day and 88% by 70th day of incubation.

Azolla-N Mineralization:

The laboratory study of *Azolla* incorporated into soil and incubated at 30°C for 25 days indicated that rate of mineralization of *Azolla* was affected by the moisture level of the soil (Fig. 19). The mineralization rate was faster at 30% SP compared with that at 100% SP, as total mineral N (NO_3+NH_4) on the average (during the incubation period) was about 1.5 time higher, being 2.82 mg N/50 g soil (56 mg N/kg soil) for 30% SP while 1.8 mg N/50 g soil (36 mg N/kg soil) for 100% SP of soil moisture.

The amount of NO_3-N formed during incubation period was about 14 times, at 30% SP as compared to 100% SP, as on the average it was 2.2 mg and 0.15 mg/50 g soil respectively. On the contrary NH_4-N formed from *Azolla*-N was about 3 times higher in soil incubated at 100% SP than at 30% SP, as on the average it was 1.6 mg and 0.6 mg N/50 g soil respectively.

The rate of mineralization of *Azolla*-N, as indicated by formation of NO_3-N in 30% SP and NH_4-N in 100% SP, was faster during the first week of incubation period. The

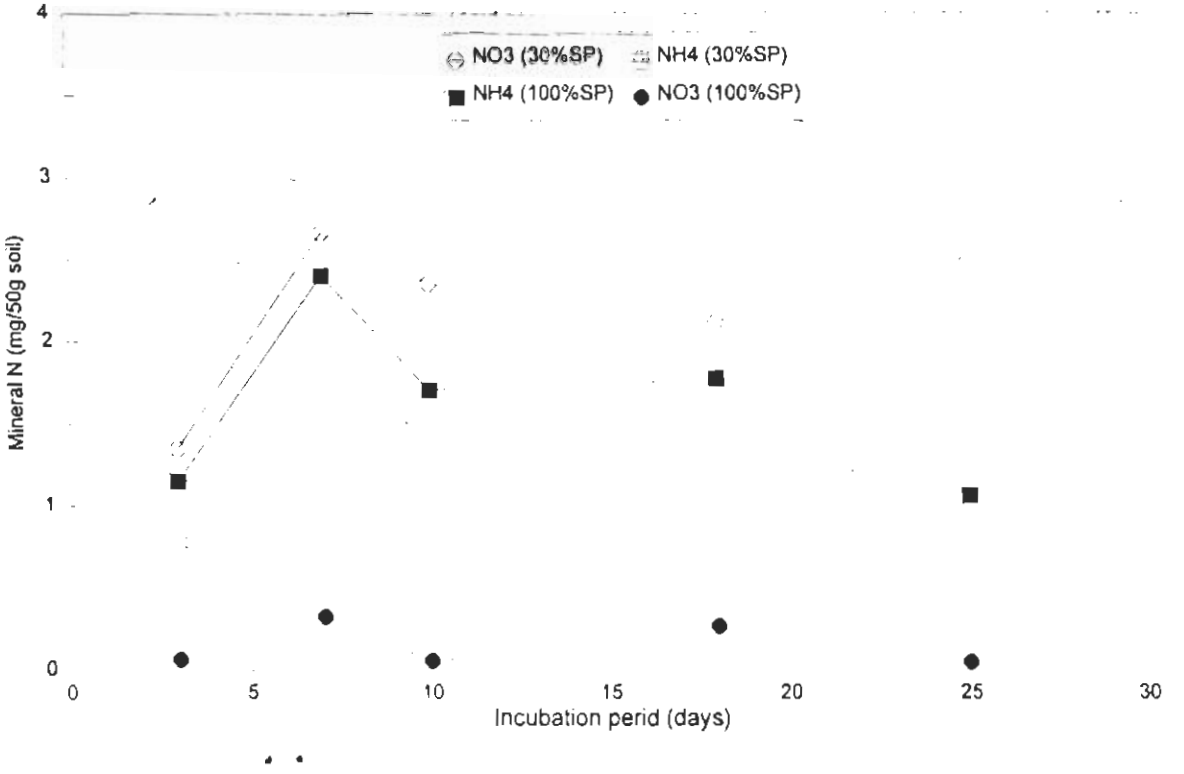

Fig. 19. Nitrogen mineralization in soil ammended with Azolla at two moisture levels. (SP = Saturation percentage).

mineralization reached to peak by the end of first week of incubation period and afterwards the rate of mineralization remained almost constant (Fig. 19).

Contribution of *Azolla* to Soil Humus:

The analysis of soil at the end of 40 days of incubation of unlabelled *Azolla* at two moisture levels showed that higher losses of organic-carbon occurred at 30% SP, moisture than at 100% SP whereas opposite situation was observed for loss of total N from the experimental soil (Table 6). At 30% SP, as compared to initial amount of organic-carbon, 41 and 37% was lost as CO_2 during incubation period for control and *Azolla* incorporated soil respectively. In case of incubation at 100% SP, these loses were 24 and 33% for control and *Azolla* incorporated soil respectively. The total N losses from the soil incubated at 30% SP was negligible being 1 & 4% for control and *Azolla* mixed soil respectively, whereas at 100% saturation, these losses were higher being 10 and 17% for control and *Azolla* incorporated soil respectively (Table 6).

During decomposition, although some of the total organic-C (added and/or of native soil) was lost as CO^2 (Table 6) as mentioned above, but most of it i.e. 59-63% for 30% SP and 67-76% for incubation at 100% SP was left undecomposed or immobilized during decomposition. Similarly, most of the N i.e. 96-99% for 30% SP and 83-90% for 100% SP. was still present in the soil (Table 6). Thus approximately double organic-C and 1.5 times nitrogen was present in *Azolla* amended soil than control.

The distribution of carbon in different organic matter fractions i.e. humic acid (HA) and fulvic acid (FA) indicated that out of total organic carbon left after decomposition in the soil, about one-third was incorporated into HA+FA and about two-third in the humin fraction. The contribution of *Azolla* incorporation into soil (25 g fwt/250 g soil) indicated that after 40 days of incubation at 30°C, almost double amount of carbon in HA+FA was present in *Azolla* amended soil than control at 30% SP incubation, being 1.91 mg and 1.14 mg/g soil, respectively.

The distribution of carbon in humic and fulvic acid indicated that for *Azolla* amended soil, it was somewhat higher (17%) in FA than in HA being 1030 and 880 mg/g soil respectively (Fig. 20a). As compared to control, almost double amount of FA was formed

Table 6. Changes in organic-carbon and total nitrogen in soil after incubation at two soil moisture levels.

Treatment		30 % saturation percent		100 % saturation percent	
	Initial	Final	loss (%)	Final	loss (%)
		Organic-carbon (mg /g soil)			
Control	5.8	3.45	*41*	4.42	*24*
Azolla	10.19	6.37	*37*	6.82	*33*
		Total nitrogen (µg /g soil)			
Control	735	730	*1*	660	*10*
Azolla	1065	1020	*4*	885	*17*

Control = Soil without Azolla.
Azolla = Soil with Azolla, mixed @25 g fwt/250 g soil.

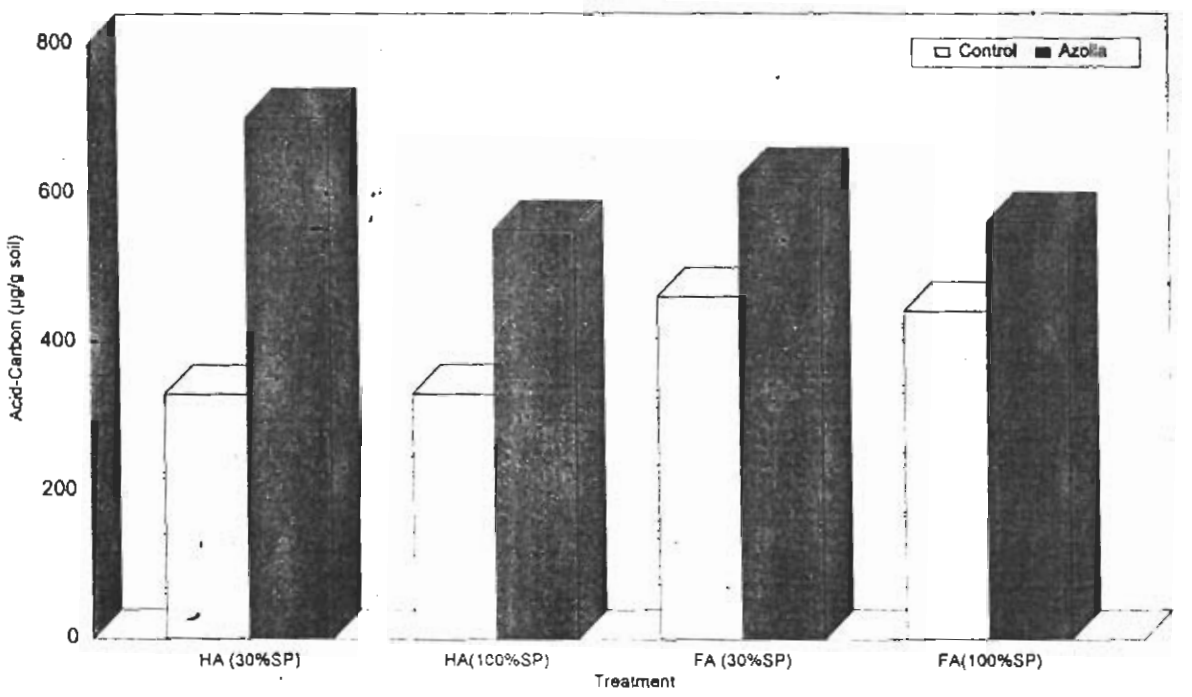

Fig. 20a. Effect of Azolla incorporation on soil humic substances,incubated at two moisture levels.
(FA=Fulvic acid, HA=Humic acid, SP=Saturation percentage)

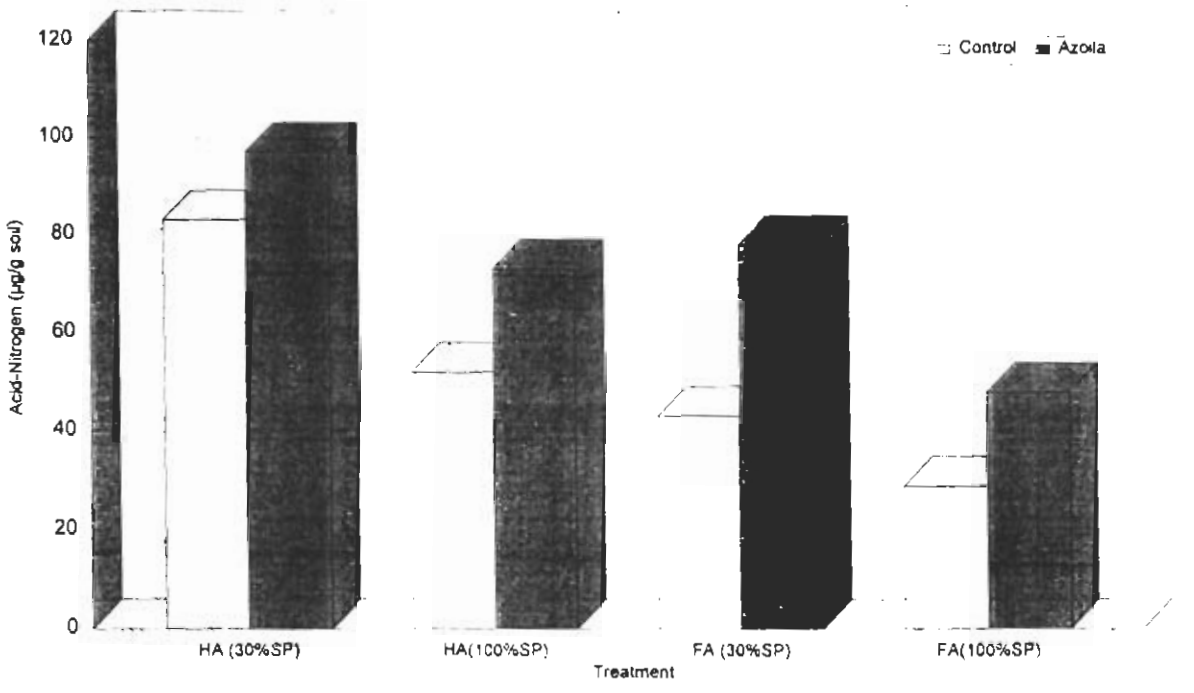

Fig. 20b.Effect of Azolla incorporation on soil humic substances,incubated at two moisture levels.
(FA=Fulvic acid, HA=Humic acid, SP=Saturation percentage)

in soil amended with *Azolla*, and higher amount of HA was formed at 30% SP than 100% SP (Fig. 20a).

The nitrogen content in HA and FA indicated that more nitrogen was immobilized in HA and FA for 30% SP than at 100% SP incubation (Fig. 20b). Higher amount of N was present in FA than in HA, especially for *Azolla* amended soil incubated at 30% SP.

USE OF *AZOLLA* IN RICE

The results of different greenhouse and field experiments using *Azolla* for increasing rice yield, tracing availability of its N to rice, improvement in fertilizer-N use efficiency are being reported in this section.

Greenhouse Studies:

Effect of *Azolla* and Blue-green Algae (Pot Expt. 1): The inoculated *Azolla* (A.p.i) grew quite fast and covered the water surface in the pot after a week or so. There was no problem of any pest during its growth and on the average it doubled its biomass every 11 days up to second week of November and thereafter its growth slowed down i.e. in the last two weeks of November and in December.

The application of *Azolla*, Blue-green algae (BGA) and nitrogen as urea on rice cv. Basmati-370 indicated that the flag leaf area was maximum for urea, followed by BGA and being minimum in control (Table 7a). The area of the second leaf was higher for chemical nitrogen applied as urea and *Azolla* inoculation (intercropped and incorporated) while it was lower and statistically similar in case of dead *Azolla*, BGA and control. The tiller height was slightly higher for urea and *Azolla* inoculation than the other treatments but the differences were not significant. The number of fertile tillers were maximum for urea followed by *Azolla* inoculation and minimum for BGA, and lower for control and dead *Azolla*. On the contrary to fertile tillers, maximum number of sterile tiller was observed for BGA while minimum for *Azolla* inoculation and urea. The panicle length was maximum for urea followed by *Azolla* and minimum for BGA and control (Table 7a).

The rice yield was maximum for urea, followed by *Azolla* and being minimum for BGA and control (Table 7b). The rice straw yield as compared to control was 60, 33, 21,

Table 7a. Effect of Azolla and blue-green algae on rice cv. Basmati-370 (Pot Expt.1).

Treatment	Flag leaf area (sq.cm)	2nd leaf area (sq.cm)	Tiller height (cm)	Fertile tillers (no./pot)	Sterile tillers (no./pot)	Panicle length (cm)
T1. Control	4.84 d	6.53 c	59	16 d	2.2 b	15.8 cd
T2. Azolla Int.	6.43 bc	7.86 b	62	17 cd	0.8 bc	16.5 bcd
T3. Azolla Int.+Med.	6.88 b	7.93 b	62	17 cd	0.4 c	16.9 bc
T4. Azolla Int.+Med.+FYM	6.40 bc	7.13 bc	61	18 bc	1.6 bc	16.7 bcd
T5. Dead Azolla+Med+FYM	6.18 bc	6.59 c	60	16 d	1.8 bc	16.6 bcd
T6. Blue-green algae+Med.	5.33 cd	6.24 c	61	14 e	3.8 a	15.5 d
T7. Blue-green algae+40kgN/ha	10.20 a	9.33 a	63	19 b	1.2 bc	18.4 a
T8. Dead Blue-green algae+60kgN/ha	10.99 a	9.66 a	63 ns	26 a	2.0 bc	17.3 ab

Int=Azolla grown as intercrop & incorporated; Med=N-free culture medium of IRRI for Azolla &BG 11 for algae;
FYM=Farmyard manure (2t/ha).
Azolla inoculum = 3 t (fwt)/ha.
Means followed by a common letter are not significantly different at the 5% level.

Table 7b. Effect of Azolla and blue-green algae on yield of Basmati-370, and total soil nitrogen after rice harvest (Pot Expt.1).

Treatment	Straw (g/pot)	Grain (g/pot)	Straw+Grain (g/pot)	Soil N (%)
T1. Control	10.2 e *0*	9.3 d *0*	19.5 d *0*	0.069 *0*
T2. Azolla Int.	11.3 d *11*	12.7 c *37*	24.1 c *24*	0.069 *1*
T3. Azolla Int.+Med.	11.2 de *10*	12.9 c *39*	24.1 c *24*	0.068 *-1*
T4. Azolla Int.+Med.+FYM	12.3 c *21*	12.7 c *36*	25.0 c *28*	0.070 *2*
T5. Dead Azolla+Med+FYM	10.4 de *2*	9.5 d *2*	19.9 d *2*	0.068 *-0*
T6. Blue-green algae+Med.	9.0 f *-11*	8.7 d *-7*	17.7 e *-9*	0.067 *-2*
T7. Blue-green algae+40kgN/ha	13.5 b *33*	14.0 b *51*	27.5 b *42*	0.068 *-1*
T8. Dead Blue-green algae+60kgN/ha	16.3 a *60*	16.9 a *82*	33.3 a *71*	0.069 *0* ns

Same as table 7a.
Figures in italics and smaller font, are % increase over control

11 and 10% higher for T8, T7, T4 and T2 respectively. It was almost equal to control for T5 (Dead *Azolla*+FYM) while minimum for BGA treatment. The rice grain yield was 82, 51, 39, 37, and 36% higher for T8, T7, T3, T2 and T4 respectively, than control. It was minimum for BGA being statistically similar with control and dead *Azolla*. The rice biomass production (straw+grain) as compared to control was 71, 42, 28, 24 and 24% higher for T8, T7, T4, T3 and T2 respectively. The rice biomass was almost equal for control and dead *Azolla* whereas minimum for BGA (Table 7b).

The total soil nitrogen, in the soil after rice harvest, was statistically similar for all the treatments, but slightly higher for *Azolla* than the remaining treatments (Table 7b).

Effect of *Azolla* and Urea (Pot Expt. 2): The rice yield of Basmati-370 was influenced by the inoculation of *Azolla* and urea (Table 8). The rice straw yield as compared to control was 29, 21, 15 and 13% higher for T5, T8, T6 and T4 respectively. It was statistically similar for control, 10 kg N/ha, *Azolla* incorporated and *Azolla* cover but minimum for *Azolla* cover.

The rice grain yield was significantly higher for *Azolla* treated pots as compared to control. It was 106, 68, 36, 29, 25, 23 and 22% higher for T6, T4, T3, T8, T2, T7 and T6 respectively, over control, however it was statistically similar for T6, T7 and control (Table 8).

The rice biomass production as indicated by straw+grain yield was higher for *Azolla* Incorporated+FYM treatment and 50 kg N/ha of urea, whereas a lower yield was observed for *Azolla* Incorporated and *Azolla* cover and minimum in control (Table 8).

Effect of *Azolla* and Farmyard Manure (Pot Expt. 3): The yield of rice was affected due to inoculation of *Azolla* as well as for the application of FYM and urea (Table 9). The higher rice straw yield was obtained for *Azolla* incorporated+FYM, urea and FYM than other treatments being 49, 47, and 31% higher than control for T6, T8 and T5 respectively.

The increase in rice grain yield, as compared to control, was 87, 73, 72, and 35% higher for T6, T8, T5, T2 respectively. Statistically similar yield was observed for *Azolla* Incorporated+P, *Azolla* Incorporated, dead *Azolla* and control, but being minimum for control (Table 9).

Table 8. Effect of Azolla and urea on yield of Basmati-370 (pot Expt.2).

Treatment	Straw (g/pot)	Grain (g/pot)	Straw+Grain (g/pot)
T1.Control	29.0 de	11.8 d	40.8 e
	0	*0*	*0*
T2.Azolla Cov.	26.8 e	14.7 c	41.4 e
	-8	*25*	*2*
T3.Azolla Int.	28.0 de	16.1 c	44.1 de
	-3	*36*	*8*
T4.Azolla Cov.+FYM(12t/ha)	32.8 bc	19.8 b	52.5 b
	13	*68*	*29*
T5.Azolla Int.+FYM	37.5 a	24.3 a	61.8 a
	29	*106*	*51*
T6.Dead Azolla	33.3 bc	14.4 cd	47.7 bcd
	15	*22*	*17*
T7.10 KgN/ ha	30.5 cd	14.5 cd	45.0 cde
	5	*23*	*10*
T8.50 KgN/ ha	35.0 ab	15.3 c	50.3 bc
	21	*29*	*23*

Cov. = Azolla grown as intercrop & not incorporated.
Int. = Azolla grown as intercrop & incorporated.
Azolla inoculum = 2 t (fwt)/ha.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

Table 9. Effect of Azolla, farmyard manure and urea on yield of Basmati-370. (pot Expt.3).

Treatment	Straw (g/pot)		Grain (g/pot)		Straw+Grain (g/pot)	
T1.Control	33.6	c	13.6	c	47.2	c
	0		*0*		*0*	
T2.Azolla Cover	27.4	d	18.4	b	45.7	c
	-19		*35*		*-3*	
T3.Azolla Int.	33.4	c	15.4	bc	48.8	c
	-1		*13*		*3*	
T4.Dead Azolla	35.2	c	15.3	bc	50.5	c
	5		*13*		*7*	
T5.Farmyard manure (6t/ha)	44.0	b	23.3	a	67.3	b
	31		*72*		*43*	
T6.Azolla Int.+FYM	49.9	a	25.3	a	75.2	a
	49		*87*		*60*	
T7.Azolla Int.+P(60kgP2O5/ha)	34.1	c	16.1	bc	50.2	c
	2		*19*		*7*	
T8.60 Kg N/ ha	49.4	a	23.4	a	72.9	a
	47		*73*		*55*	

Int. = Azolla grown as intercrop & incorporated.

Azolla inoculum = 2t (fwt)/ha.

Means followed by a common letter are not significantly different at the 5% level.

Figures in italics and smaller font, are % increase over control.

The rice biomass (straw + grain) yield pattern showed higher yield for *Azolla*+FYM and 60 kg N/ha treatment, followed by FYM and being lesser for the remaining treatments (Table 9).

Effect of *Azolla* on Fertilizer-N Use Efficiency (Pot Expt. 4): Some of the results of this experiment has already been reported (Ali and Malik, 1988) and the details are given here. During rice growth period it was observed that *Azolla* grew without any problem in the inoculated pots. The algal growth comprising mainly of blue-green algae and diatoms, was observed in the floodwater and on the soil surface in control and only urea treated pots; whereas almost no algal growth was found in pots having *Azolla*, and floodwater was clear.

The difference in floodwater temperature was maximum at noon, and was 31.0, 30.2, 30.0 and 29.6°C in control, urea, *Azolla* incorporated and *Azolla* cover pots, respectively.

The average of readings of floodwater pH recorded between 56-111 DAT showed a large variation during day time, and as compared to morning it increased during noon time especially in control and only urea treated pots than in pots having *Azolla* alone or with urea (Fig. 21). The floodwater pH in control pots rose to 8.2 by 11 am, and reached to 8.6 at 1 pm and remained around 8.5 upto 3 pm. Similarly the pH remained high (8.3) from 12 to 3 pm in only urea treated pots, while the pH in urea + *Azolla* treatment was lower (7.8) at 10 am and also remained low during noon time. The lowest value for floodwater pH was for urea + *Azolla* cover treatment being 7.4 at 3 pm, and highest in control being 8.6 during noon, indicating a difference of 1.2 units.

The pH at soil-water interface was 7.9, 7.5, 7.4 and 7.1 for control, urea, *Azolla* cover and *Azolla* incorporated treated pots at noon time respectively. Similarly the pH of top 3-5 cm soil was higher in control than *Azolla* cover, urea and *Azolla* incorporated pots, being 7.6, 7.3, 7.1 and 7.0 respectively.

The maximum tiller height was 63, 64, 64, 64, 71, 70, 68, 67 and 72 for T1 to T9 respectively, and was significantly higher for urea at 60 kg N/ha than the remaining treatments. The total tillers per pot were 12, 14, 13, 18, 17, 18, 19, 21 and 21 for T1 to T9 respectively, being significantly higher for urea+*Azolla* incorporated treatments as compared to other treatments.

The rice straw, grain and straw+grain yield (Table 10a) showed that straw yield was significantly higher for urea than control or *Azolla* alone. It was 68% and 75% higher for

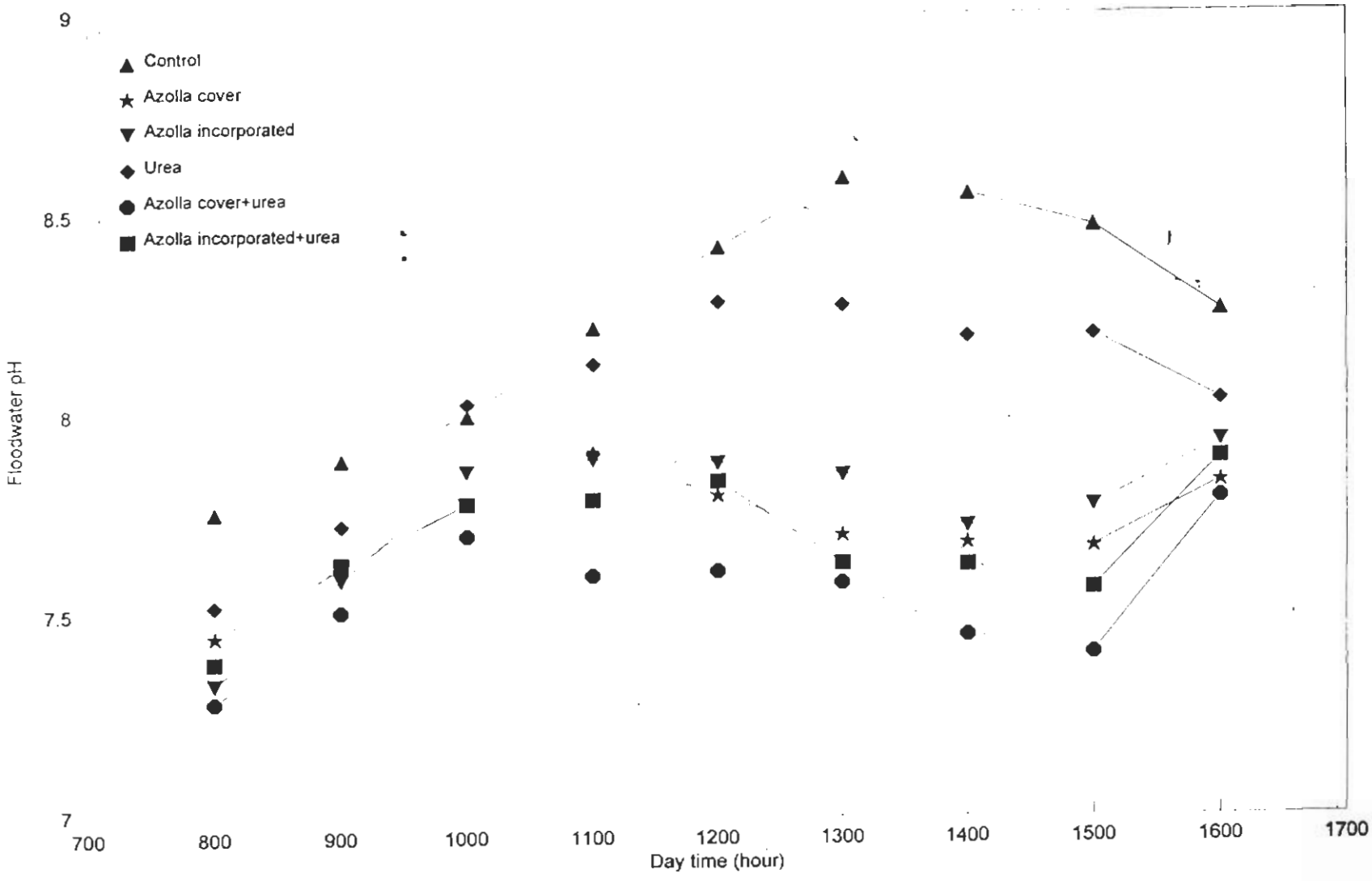

Fig. 21. Kinetics of floodwater pH during day time in rice-Azolla culture in a pot experiment.

Table 10a. Effect of Azolla and urea on rice yield cv.IR-6 (pot Expt.4).

Treatment	Straw g/pot	Grain g/pot	Straw+Grain g/pot
T1.Control	12.24 b	11.12 d	23.36 d
	0	*0*	*0*
T2.Azolla Cov.	12.20 b	14.39 cd	26.59 d
	-0	*29*	*14*
T3.Azolla Int.	11.79 b	18.77 bc	30.56 cd
	-4	*69*	*31*
T4.30 kgN/ha	19.68 a	16.48 cd	36.16 bc
	61	*48*	*55*
T5.60 kgN/ha	20.56 a	23.86 ab	44.42 ab
	68	*115*	*90*
T6.Azolla Cov.+30 kgN/ha	17.74 a	18.60 bc	36.34 bc
	45	*67*	*56*
T7.Azolla Cov.+60 kgN/ha	18.71 a	23.64 ab	42.35 ab
	53	*113*	*81*
T8.Azolla Int.+30 kgN/ha	21.37 a	23.46 ab	44.84 ab
	75	*111*	*92*
T9.Azolla Int.+60 kgN/ha	20.62 a	28.47 a	49.09 a
	69	*156*	*110*

Cov. = Azolla grown as intercrop & not incorporated.
Int. = Azolla grown as intercrop & incorporated.
Azolla inoculum = 3 t (fwt)/ha.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

Table 10b. Effect of Azolla and urea on total nitrogen yield (g/pot) in rice and soil after rice harvest (Pot Expt.4).

Treatment	Straw	Grain	Soil	Straw +Grain	Plant +Soil
T1.Control	0.047 b	0.082 e	6.21 b	0.129 f	6.34 d
	0	*0*	*0*	*0*	*0*
T2.Azolla Cov.	0.050 b	0.111 de	6.56 ab	0.161 ef	6.72 abcd
	6	*35*	*6*	*24*	*6*
T3.Azolla Int.	0.047 b	0.164 cd	6.70 a	0.211 cde	6.91 ab
	-2	*100*	*8*	*63*	*9*
T4.30 kgN/ha	0.083 a	0.120 de	6.25 b	0.203 de	6.46 cd
	76	*46*	*1*	*57*	*2*
T5.60 kgN/ha	0.076 a	0.201 bc	6.25 b	0.276 ab	6.53 bcd
	60	*145*	*1*	*113*	*3*
T6.Azolla Cov.+30 kgN/ha	0.070 a	0.158 cd	6.84 a	0.228 bcd	7.06 a
	48	*92*	*10*	*76*	*11*
T7.Azolla Cov.+60 kgN/ha	0.070 a	0.217 ab	6.58 ab	0.287 ab	6.87 abc
	48	*164*	*6*	*122*	*8*
T8.Azolla Int.+30 kgN/ha	0.080 a	0.185 bc	6.57 ab	0.265 bc	6.83 abc
	68	*125*	*6*	*105*	*7*
T9.Azolla Int.+60 kgN/ha	0.077 a	0.259 a	6.53 ab	0.336 a	6.87 abc
	63	*215*	*5*	*159*	*8*

Cov. = Azolla grown as intercrop & not incorporated.
Int. = Azolla grown as intercrop & incorporated.
Azolla inoculum = 3 t (fwt)/ha.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

Table 10c. Effect of Azolla on the fertilizer N recovery (%) of the N-15 labelled urea in rice and soil after rice (Pot Expt.4).

Treatment	Straw	Grain	Soil	Straw +Grain	Plant +Soil
T4.30 kgN/ha	8.9 a	13.4 c	36.1 bc	22.2 b	58.3 b
T5.60 kgN/ha	6.3 b	20.2 b	32.4 c	26.4 ab	58.8 b
T6.Azolla Cov.+30 kgN/ha	8.1 ab	16.3 bc	44.4 abc	24.4 b	68.8 ab
T7.Azolla Cov.+60 kgN/ha	6.6 b	22.1 ab	52.7 a	28.7 ab	81.4 a
T8.Azolla Int.+30 kgN/ha	9.6 a	18.7 bc	48.4 ab	28.3 ab	76.7 a
T9.Azolla Int.+60 kgN/ha	6.8 b	26.6 a	35.1 bc	33.4 a	68.5 ab

Same as table 10b

60 kg N/ha and 60 kg N/ha + *Azolla* incorporated treatments respectively. The rice grain yield showed a slightly different pattern than straw yield, and as compared to control it was 111-156% higher for *Azolla* incorporated + urea applied at 30 or 60 kg N/ha and 60 kg N/ha urea alone or with *Azolla* cover as observed in T5 and T7-T9 (Table 10a). A significantly higher grain yield was observed for *Azolla* cover and *Azolla* incorporated treatment, and was 29 and 69% higher for T2 and T3 respectively, than control. The rice biomass (straw+grain) showed a general trend of grain yield and was higher for *Azolla* + urea treatment and also for higher rate of nitrogen fertilizer application.

The total N uptake in rice and retention in soil (Fig. 10b) showed that in rice straw it was generally higher for urea applied pots. The total nitrogen uptake in grain showed a pattern similar to grain yield for different treatments. The retention of nitrogen in the soil was higher for *Azolla* alone or *Azolla*+urea treatments than the control or urea application either at 30 or 60 kg N/ha. In rice plant (straw+grain) the nitrogen uptake was of similar pattern as of rice grain. The total nitrogen in rice plant+soil was significantly higher for *Azolla* alone and *Azolla*+urea treatments than control or only urea treatments.

The recovery of ^{15}N labelled urea in rice and soil after rice harvest (Table 10c) showed that in rice straw it was lower for 60 kg N/ha than 30 kg N/ha whereas the situation reversed for recovery in grain and was higher for higher rate of urea application, and was further improved in the presence of *Azolla*. The retention of ^{15}N labelled urea in soil was generally higher for urea+*Azolla* than urea only treatments. The ^{15}N recovery of applied urea in rice plant (straw+grain) was higher for higher rate of urea application alone or with *Azolla*. The total fertilizer-N recovery in plant+soil was higher (69-81%) for urea+*Azolla* treatments than urea only having 58-59% recovery.

Field Studies:

Effect of *Azolla* and Urea (Field Expt. 1): It was observed that *Azolla* growth was different in different plots. The average fresh biomass recorded on first October with a quadrat was 750, 930 and 1184 g/m^2 for T2, T4 and T3 respectively. The dry matter yield of rice (Table 11) showed that higher straw yield was obtained for urea application than the remaining treatments. As compared to control the increase in grain yield was 20, 10, 8 and 5% for *Azolla* + FYM, *Azolla*+10 kg N/ha, *Azolla* and 60 kg N/ha respectively. The rice

Table 11. Effect of Azolla and urea on yield of Basmati-370 (Field Expt.1).

Treatment	Straw (kg/ha)	Grain (kg/ha)	Straw+Grain (kg/ha)
T1.Control	6074 ab	2650 b	8724
	0	*0*	*0*
T2.Azolla Int.	6098 ab	2869 ab	8966
	0	*8*	*3*
T3.Azolla Int.+FYM (12t/ha)	6059 ab	3186 a	9245
	-0	*20*	*6*
T4.Azolla+10 KgN/ha	6637 ab	2920 ab	9557
	9	*10*	*10*
T5.60 KgN/ha	6917 a	2772 b	9689
	14	*5*	*11*
			ns

Int.=Azolla grown as intercrop & incorporated.
Azolla inoculum = 0.1 t (fwt)/ha.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

biomass (straw+grain) was higher for both the urea treatments than the remaining treatments.

Effect of *Azolla* and Farmyard Manure (Field Expt. 2): The rice yield (Table 12) showed that rice straw yield was higher for urea, *Azolla*+FYM and FYM treatments than the control and *Azolla* only treatment. The grain yield was 10, 21, 27 and 56% higher than control for *Azolla*, FYM, *Azolla*+FYM and 60 kg N/ha treatment respectively. The total rice biomass also followed the trend of grain yield.

Evaluation of *Azolla*-N Uptake and Yield Response (Field Expt. 3): The average soil temperature at 5 cm depth during rice and wheat cropping period (Fig. 22) showed that after rice transplanting during third week of July it gradually decreased during rice growth period. The average soil temperature was 37, 34, 33, 26 and 18°C during July, August, September, October and November respectively.

The maximum tiller height, total tillers and fertile tillers were in the increasing order of control < *Azolla* < urea.

The rice yield (Table 13a) showed that straw yield was significantly higher for urea than control and *Azolla* treatment. Similarly the overall grain yield was higher for urea treatments, however, for *Azolla* treatment it was 11-13% higher than control. As compared to control the total rice biomass was 68-77% higher for urea application treatments and 8-10% for *Azolla* incorporation treatment.

The ^{15}N recovery from labelled *Azolla* and urea indicated that in general it was higher for urea than *Azolla* treatments (Table 13b). It was observed that ^{15}N recovery from *Azolla*, incorporated at 40 DAT, was higher in straw as well as in grain than its incorporation at 80 DAT. Similarly the ^{15}N recovery of labelled urea was higher in rice grain when it was applied at 40 DAT than 80 DAT.

Evaluation of *Azolla*-N Uptake and Yield Response (Field Expt. 4): The maximum tiller height, total tillers and fertile tillers showed statistically similar values for *Azolla* and urea treatments, but were significantly higher than control.

The rice yield (Table 14) showed that it was generally higher for urea than *Azolla* treatments. As compared to control the increase in straw yield was 18-22% for *Azolla* and 32-46% for urea, while for grain yield it was 17-21% for *Azolla* and 70-83% for urea

Table 12. Effect of Azolla and farmyard manure on yield of Basmati-370. (Field Expt.2).

Treatment	Straw (kg/ha)		Grain (kg/ha)		Straw+Grain (kg/ha)	
T1.Control	4454	d	1932	d	6386	d
	0		*0*		*0*	
T2.Azolla Int.	4747	cd	2125	cd	6872	cd
	7		*10*		*8*	
T3.FYM (6 t/ha)	5399	bc	2343	bc	7742	bc
	21		*21*		*21*	
T4.Azolla Int.+FYM(6 t/ha)	5652	b	2452	b	8105	b
	27		*27*		*27*	
T5.60 KgN/ha	6952	a	3016	a	9968	a
	56		*56*		*56*	

Int. = Azolla grown as intercrop & incorporated.
Azolla inoculum = 6 t (fwt)/ha.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

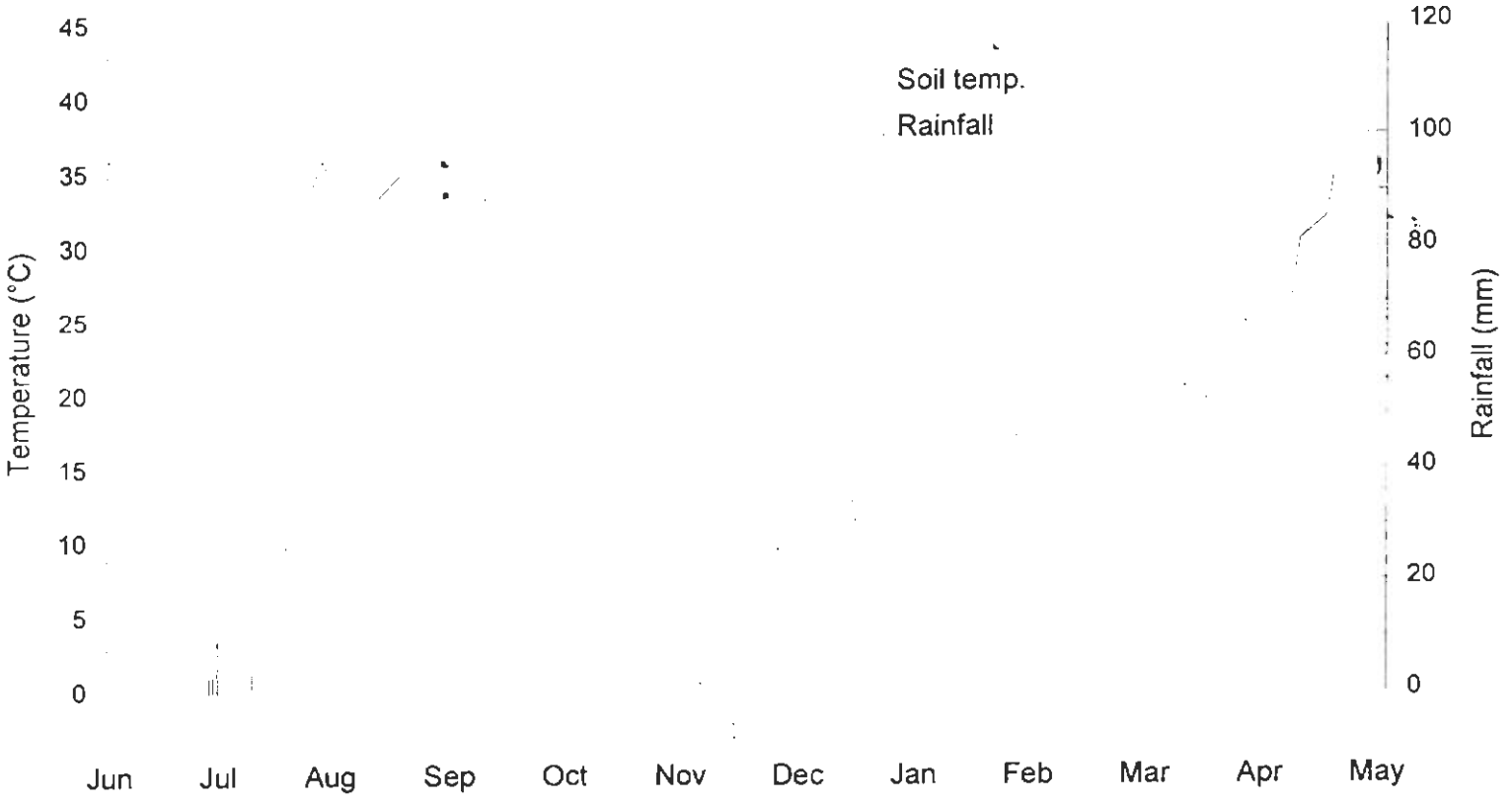

Fig.22. Soil temperature at 5cm depth in experimental plots during rice-wheat cropping period.

Table 13a. Effect of Azolla and urea on rice yield cv.IR-6 (Field Expt.3).

Treatment	Straw kg/ha		Grain kg/ha		Straw+Grain kg/ha	
T1. Control	1368	b	1718	b	3085	b
	0		*0*		*0*	
T2.Azolla: 30 N15+30 N14	1461	b	1940	b	3401	b
	7		*13*		*10*	
T3.Azolla: 30 N14+30 N15	1441	b	1906	b	3347	b
	5		*11*		*8*	
T4.Urea: 30 N15+30 N14	2767	a	2680	a	5447	a
	102		*56*		*77*	
T5.Urea: 30 N14+30 N15	2622	a	2570	a	5191	a
	92		*50*		*68*	
T6.Urea: 20 N15+40 N15	2561	a	2673	a	5234	a
	87		*56*		*70*	

Azolla or urea, ordinary (N14) or labelled (N15) was incorporated at 40 and 80 DAT.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

Table 13b. Effect of Azolla and urea on the nitrogen recovery (%) in rice. (Field Expt. 3).

Treatment	Straw		Grain		Straw+Grain	
T2.Azolla: 30 N15+30 N14	4.3	b	13.4	b	17.8	b
T3.Azolla: 30 N14+30 N15	2.8	b	9.1	b	11.8	b
T4.Urea: 30 N15+30 N14	11.4	a	23.8	a	35.2	a
T5.Urea: 30 N14+30 N15	11.6	a	28.7	a	40.4	a
T6.Urea: 20 N15+40 N15	12.0	a	28.4	a	40.4	a

Same as table 13a.

Table 14. Effect of Azolla and urea on rice yield cv.IR-6 (Field Expt.4).

Treatment	Straw kg/ha		Grain kg/ha		Straw+Grain kg/ha	
T1.Control	4040	c	1849	b	5888	c
	0		*0*		*0*	
T2.Azolla: 30 N15+30 N14	4754	bc	2165	b	6919	bc
	18		*17*		*18*	
T3.Azolla: 30 N14+30 N15	4945	b	2239	b	7184	b
	22		*21*		*22*	
T4.Urea: 30 N15+30 N14	5322	ab	3233	a	8555	a
	32		*75*		*45*	
T5.Urea: 30 N14+30 N15	5763	a	3149	a	8912	a
	43		*70*		*51*	
T6.Urea: 20 N15+40 N15	5893	a	3376	a	9268	a
	46		*83*		*57*	

Azolla or urea ,ordinary (N14) or labelled (N15) was incorporated at 40 and 80 DAT.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

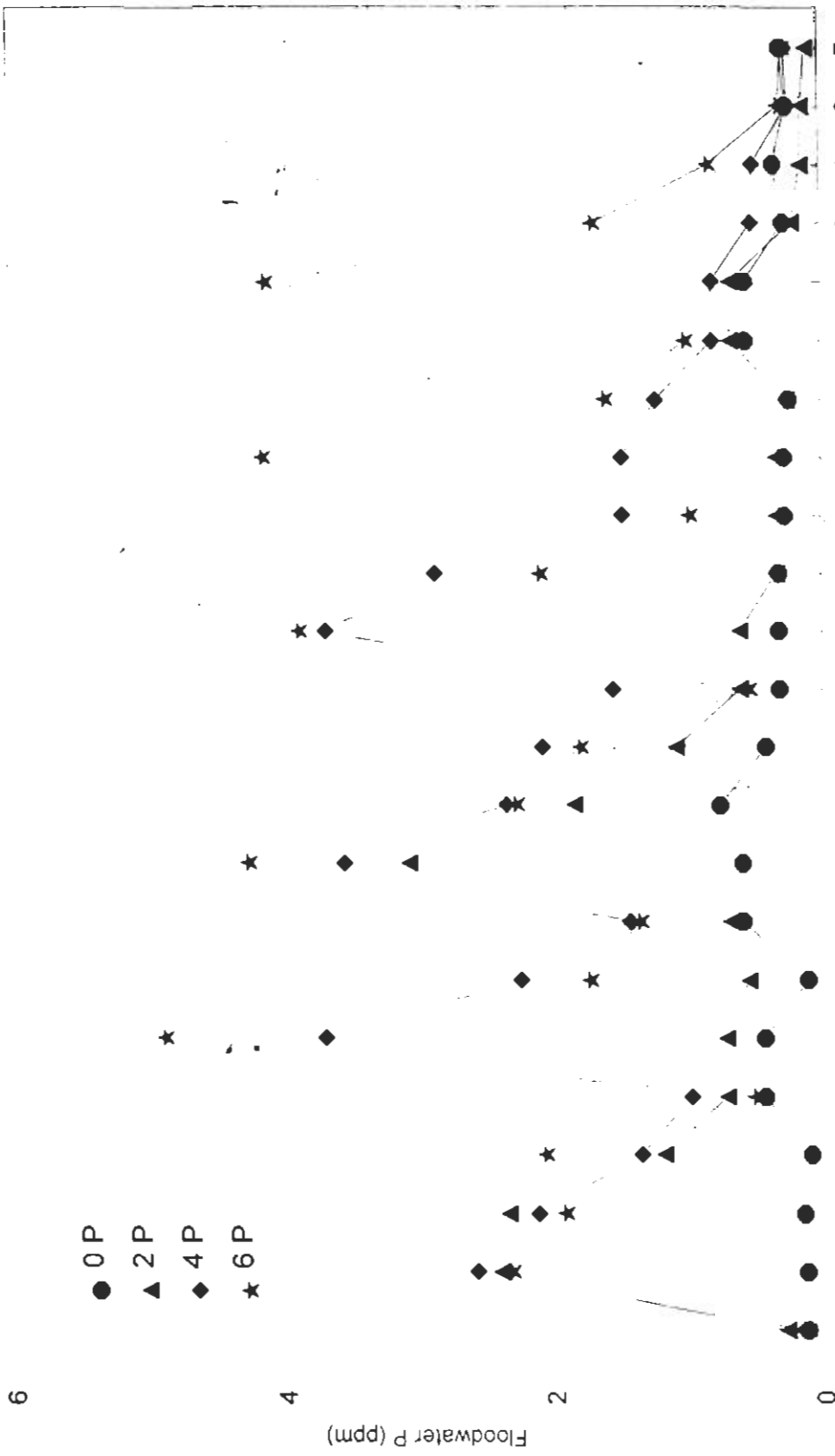

Fig. 23. Kinetics of floodwater P during P applications in Azolla nursery plots.
(On the same date the 1st point is before while the 2nd one after P applcation)

treatments. Similarly the total rice biomass was higher for urea than *Azolla* but *Azolla* plots have 18-22% higher rice biomass than control.

The ^{15}N recovery in rice plant (not shown) from *Azolla* and urea was 16-27% for *Azolla* and 20-45% for urea treatments. The ^{15}N recovery in rice was higher (9%) from *Azolla* incorporated at 10 DAT than incorporated at 40 DAT with a value of 4%. On the contrary the ^{15}N recovery of urea was higher (12%) when applied at 40 DAT than at 10 DAT, being 7%. In grain the ^{15}N recovery was 18, 13, 14, 33 and 33 for T2 to T6 respectively. The ^{15}N recovery in straw+grain was 27, 16, 20, 45 and 44% for T2 to T6 respectively.

Effect of P Enriched *Azolla* on N Availability (Field Expt. 5): The bioassay of seven different soils tested for P response for *Azolla* growth showed that only one soil produced about 35% higher biomass for P application than control. Therefore, this soil was selected for growing *Azolla* nursery.

During *Azolla* growth in nursery, P was applied at four levels and the floodwater P was monitored. It was observed that with the frequent application of P into floodwater its P level can be raised than equilibrium level (Fig. 23). The period of higher level of floodwater P than control, was dependent on the frequency of P application. It remained higher during *Azolla* growth and was in the increasing order of 0, 2, 4 and 6 P treatments. The figure shows that as soon as P application is stopped the floodwater P level reaches to equilibrium level within a week.

Azolla growth remained poor in all these 4 nurseries and did not improve even with the application of macro and micronutrients. Thus due to poor *Azolla* growth insufficient *Azolla* providing only 4-5 kg N/ha was obtained and was incorporated into experimental plots. The *Azolla* harvested at the end of the nursery growth and incorporated 40 DAT into experimental plots, did not show much difference in N content for different P applications and had 2.08, 2.62, 2.56 and 2.75% N on dry weight basis for 0, 2, 4 and 6 P *Azolla* nursery respectively.

The number of fertile tillers per plot were lesser for control and *Azolla* than urea treatment, and increased in number with an increase in amount of N applied as urea, and was 35% higher for 45 kg N/ha than control.

The rice yield (Table 15a) showed that as compared to control it was slightly higher for *Azolla*, but more for urea treatments especially at higher doses of N application. The rice straw yield was upto 16% higher for *Azolla* and upto 22% for urea than control. Similarly rice grain yield increased upto 11% for *Azolla* and 21% for urea as compared to control. The total rice biomass and grain yield increased with an increase in urea-N application from 15 to 30 kg N/ha but this increase was not significant for increase from 30 to 45 kg N/ha application.

The ^{15}N recovery in rice straw was about 3% for urea and was 2-3% for *Azolla*, while in grain it was 10-12% for urea and 6-11% for *Azolla*. In rice plant (straw+grain) the ^{15}N recovery from both these sources ranged between 11-15% of the applied N as urea or *Azolla*.

Effect of *Azolla* on Urea ^{15}N Recovery (Field Expt. 6): The floodwater pH of experimental plots recorded during 1-3 p.m. indicated that it was higher for non-*Azolla* treatments than *Azolla* treatments (Fig. 24). The pH was maximum (9.6) for T5 having urea broadcasted onto floodwater and was minimum (7.9) for T3 having urea incorporated and *Azolla* as cover, thus a maximum difference of 1.7 units was observed in these experimental plots.

The rice yield (Table 16a) in these plots showed that straw yield was higher for urea incorporated+*Azolla* and urea incorporated treatments as compared to other treatments. The increase in rice grain yield was 20-23% for 30 kg N/ha of urea application at 2 weeks after transplanting (T5-T7), and 13% for *Azolla* incorporation (T4). The root biomass was statistically similar for all the treatments, however, it was 6-22% higher than control in the remaining treatments. The straw+grain and straw+grain+root biomass was generally higher for urea and *Azolla* treatment than control and was maximum for urea incorporated+*Azolla* cover treatments (T3 and T6).

The total nitrogen uptake in rice and retention in soil after rice harvest (Table 16b) indicated that it was higher in rice straw for urea incorporated at 20 and 60 kg N/ha alone (T2 and T8) or urea+*Azolla* (T5, T6 and T8). In grain the total N uptake was higher for T5-T7 having 30 kg N/ha of urea alone and in combination with *Azolla*. The N in whole plant (straw+grain+root) was higher for T3 and T5-T7 having urea+*Azolla* combinations.

Table 15a. Effect of Azolla and urea on the dry matter yield (kg/ha) of IR-6 rice. (Field Expt.5).

Treatment	Straw		Grain		Straw+Grain	
T1. Control	1995	b	2699	bc	4693	bc
	0		*0*		*0*	
T2. Urea 15N	2254	ab	2747	abc	5001	abc
	13		*2*		*7*	
T3. Urea 30N	2407	a	3225	ab	5631	a
	21		*19*		*20*	
T4. Urea 45N	2436	a	3255	a	5691	a
	22		*21*		*21*	
T5. Azolla 0P	2017	b	2579	abc	4595	c
	1		*-4*		*-4*	
T6. Azolla 2P	2319	ab	2922	abc	5241	abc
	16		*8*		*12*	
T7. Azolla 4P	2310	ab	2994	ab	5304	ab
	16		*11*		*13*	
T8. Azolla 6P	2054	b	2692	bc	4746	bc
	3		*0*		*1*	

Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control

Table 15b. Effect of Azolla and urea on the nitrogen recovery (%) in rice. (Field Expt.5).

Treatment	Straw		Grain		Straw+Grain	
T2. Urea 15N	2.89	ab	9.68	ab	12.57	ab
T3. Urea 30N	3.17	a	11.56	a	14.73	a
T4. Urea 45N	2.88	ab	11.25	a	14.13	a
T5. Azolla 0P	1.73	c	6.68	c	8.41	c
T6. Azolla 2P	2.81	ab	11.15	a	13.96	a
T7. Azolla 4P	2.60	b	10.44	ab	13.04	ab
T8. Azolla 6P	2.12	c	8.75	b	10.87	b

Same as table 15a.

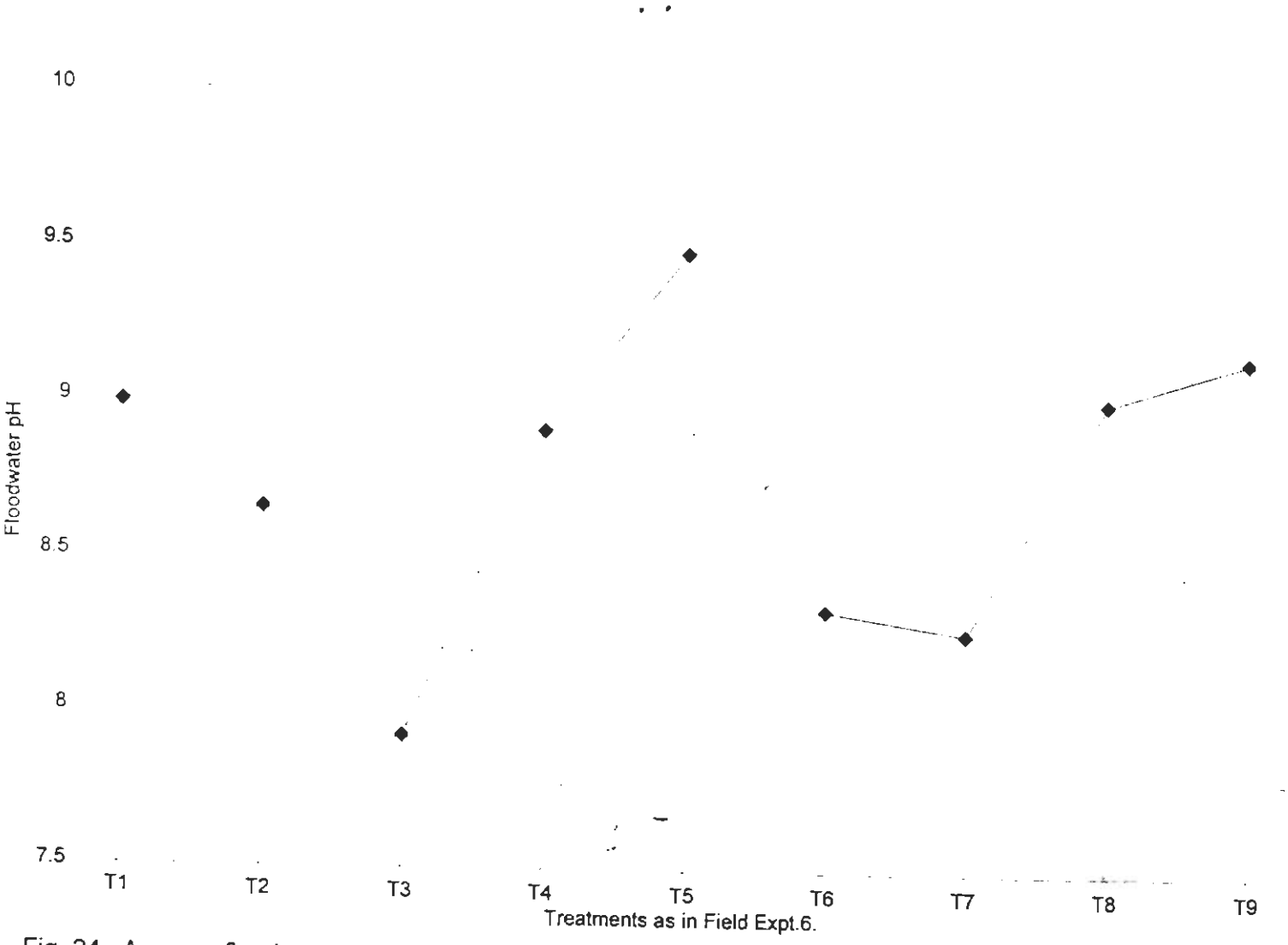

Fig. 24. Average floodwater pH during noon (1-3 pm) in rice-Azolla cuture; in isotope plots.
(pH for T1-T4 after urea incorporation while for T5-T9 after urea broadcasting)

Table 16a. Effect of Azolla and urea on the dry matter yield (kg/ha) of IR-6 rice.(Field Expt.6).

Treatment	Straw		Grain		Root	Straw+Grain		Straw+Grain +Root	
T1.Control	1660 *0*	d	2431 *0*	ab	641 *0*	4091 *0*	b	4731 *0*	bc
T2. Urea 30N Incor. T	2100 *27*	abc	2594 *7*	ab	772 *20*	4694 *15*	ab	5466 *16*	abc
T3. Urea 30N Incor. T+ Az.Cov.T	2379 *43*	a	2599 *7*	ab	784 *22*	4978 *22*	a	5763 *22*	a
T4. Az. 30 N Incor. T	1951 *18*	bcd	2743 *13*	ab	677 *6*	4694 *15*	ab	5371 *14*	abc
T5. Urea 30 N BC. 2W	1833 *10*	cd	2910 *20*	a	778 *21*	4744 *16*	ab	5522 *17*	abc
T6. Urea 30 N BC. 2W+ Az.Cov.T	1917 *16*	bcd	2986 *23*	a	756 *18*	4903 *20*	a	5659 *20*	ab
T7.Urea30NBC.2W+Az.-Cov.T+Az.Incor.4W	1530 *-8*	d	2954 *22*	a	759 *19*	4484 *10*	ab	5244 *11*	abc
T8. Urea 60 N Incor. T	2321 *40*	ab	2217 *-9*	b	763 *19*	4538 *11*	ab	5300 *12*	abc
T9.Az.Cov. T	2119 *28*	abc	2178 *-10*	b	703 *10* ns	4297 *5*	ab	5000 *6*	abc

T. = at rice transplanting, BC. = broadcasted, W. = week after rice transplanting.
Incor. = incorporated into mud. Cov. = Azolla grown as intercrop & not incorporated.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control

Table 16b. Effect of Azolla and urea on the total N yield (kg/ha) of rice cv. IR-6 and soil. (Field Expt.6.).

Treatment	Straw		Grain		Root	Straw+Grain		Straw+Grain +Root	
T1.Control	6.72 *0*	bc	22 *0*	c	2.41 *0*	29 *0*	c	31 *0*	c
T2. Urea 30N Incor. T	8.16 *21*	ab	23 *3*	bc	2.74 *14*	31 *7*	bc	34 *8*	bc
T3. Urea 30N Incor. T+ Az.Cov.T	9.18 *37*	a	25 *13*	bc	2.92 *21*	34 *18*	abc	37 *18*	abc
T4. Az. 30 N Incor. T	7.81 *16*	ab	20 *-9*	c	2.53 *5*	28 *-3*	c	31 *-2*	c
T5. Urea 30 N BC. 2W	8.16 *21*	ab	30 *34*	ab	3.10 *29*	38 *31*	ab	41 *31*	ab
T6. Urea 30 N BC. 2W+ Az.Cov.T	8.26 *23*	ab	33 *47*	a	3.13 *30*	41 *41*	a	44 *41*	a
T7. Urea30NBC.2W+Az.-Cov.T+Az.Incor.4W	5.82 *-13*	c	30 *35*	ab	2.89 *20*	36 *24*	abc	39 *23*	abc
T8. Urea 60 N Incor. T	8.81 *31*	a	20 *-12*	c	2.75 *14*	29 *-2*	c	31 *-0*	c
T9. Az.Cov. T	8.59 *28*	ab	22 *0*	c	3.00 *24* ns	31 *7*	bc	34 *8*	bc

T. = at rice transplanting, BC. = broadcasted, W. = week after rice transplanting.
Incor. = incorporated into mud. Cov. = Azolla grown as intercrop & not incorporated.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control

c. Effect of Azolla and urea on the nitrogen recovery (%) in rice cv. IR-6 at

nt	Straw		Grain		Root		Soil after rice				Straw+Grain		Root+Soil		Plant+Soil	
							0-15cm		15-30cm							
30N Incor. T	2.34	e	8	b	0.93	b	26	bc	4.39	abc	10	c	32	bc	42	cd
30N Incor. T+ Cov.T	4.31	bc	11	b	1.36	a	34	bc	3.97	bc	15	b	40	bc	55	bc
30 N Incor. T	2.98	de	7	b	0.83	b	73	a	4.54	abc	10	c	78	a	88	a
30 N BC. 2W	5.55	a	20	a	1.61	a	29	bc	5.64	a	26	a	36	bc	62	b
30 N BC. 2W+ Cov.T	5.15	ab	21	a	1.50	a	38	b	5.43	ab	26	a	45	b	71	ab
30NBC.2W+Az.-T+Az.Incor.4W	3.53	cd	20	a	1.38	a	35	bc	3.34	c	24	a	40	bc	64	b
60 N Incor. T	3.26	cde	7	b	0.98	b	20	c	4.81	abc	10	c	25	c	36	d

e transplanting, BC. = broadcasted, W. = week after rice transplanting.
ncorporated into mud. Cov. = Azolla grown as intercrop & not incorporated.
llowed by a common letter are not significantly different at the 5% level.

The ^{15}N recovery from labelled urea and *Azolla* (Table 16c) indicated that in rice straw it was higher for urea application and urea+*Azolla* treatment i.e. T5 and T6. In rice grain and root, higher ^{15}N recovery was observed for T5-T7, having urea application 14 DAT alone or with *Azolla* cover.

The ^{15}N recovery in soil after rice was 20-73% in top (0-15 cm) soil while only 3-6% was recovered in bottom zone (15-30 cm). In top plough layer it was maximum (73%) for *Azolla* incorporated treatment (T4) and ranged 20-38% for urea treatments alone or with *Azolla* cover. The ^{15}N recovery in above soil parts of rice i.e. straw+grain showed a similar trend to that of grain and root. In the underground part of rice+soil the ^{15}N recovery was higher for *Azolla* incorporated than the other treatments. In the plant+soil the ^{15}N recovery was higher for *Azolla* incorporated and 30 kg N/ha application at 14 DAT, than the remaining treatments; and it was generally lower for urea than urea+*Azolla* treatments.

RESIDUAL EFFECT OF *AZOLLA* ON WHEAT

The residual effect of *Azolla* and urea applied to rice on the subsequent wheat crop was studied in greenhouse as well as under field conditions.

Greenhouse Studies:

Pot Experiment 1R: The yield of wheat (Table 17), grown after Basmati-370 rice experiment 2, indicated that wheat straw yield was significantly higher for *Azolla* incorporated and *Azolla* incorporated+FYM treatment (T3 and T5). As compared to control, the wheat grain yield was significantly higher (23-30%) for *Azolla* incorporated (T3) and *Azolla* incorporated+FYM (T5), whereas it was 6-10% higher for urea application at 10 and 50 kg N/ha. The total wheat biomass was 28-31% higher for *Azolla* incorporated and *Azolla* incorporated+FYM than control.

Pot Experiment 2R: The wheat grown after IR-6 rice experiment 4, indicated that wheat straw and grain yield was generally higher for *Azolla*+urea treatment than urea only (Table 18). The grain yield was 15% higher for *Azolla* cover and upto 34% for *Azolla*

Table 17. Residual effect of Azolla and urea on wheat, grown after Basmati-370 of pot Expt.2. (pot Expt.-1R).

Treatment (given to preceeding rice)	Straw (g/pot)		Grain (g/pot)		Straw+Grain (g/pot)	
T1.Control	6.2	b	5.7	c	11.9	b
	0		*0*		*0*	
T2.Azolla Cov.	6.7	b	6.3	bc	13.0	b
	8		*10*		*9*	
T3.Azolla Int.	8.1	a	7.1	ab	15.2	a
	31		*23*		*28*	
T4.Azolla Cov.+FYM(12t/ha)	6.6	b	6.3	bc	12.9	b
	6		*11*		*8*	
T5.Azolla Int.+FYM	8.2	a	7.4	a	15.6	a
	32		*30*		*31*	
T6.Dead Azolla	7.0	ab	6.1	bc	13.1	b
	13		*7*		*10*	
T7.10 KgN/ ha	6.7	b	6.0	c	12.7	b
	8		*6*		*7*	
T8.50 KgN/ ha	6.8	b	6.3	bc	13.0	b
	10		*10*		*10*	

Cov. = Azolla grown as intercrop & not incorporated.
Int. = Azolla grown as intercrop & incorporated.
Azolla inoculum = 2 t (fwt)/ha.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

Table 18. Residual effect of Azolla and urea on wheat sown after rice of pot Expt. 4 (pot Expt.2R).

Treatment (given to preceding rice)	Straw g/pot	Grain g/pot	Straw + Grain g/pot
T1.Control	10.44	7.46	17.90
	0	*0*	*0*
T2.Azolla Cov.	10.85	8.55	19.40
	4	*15*	*8*
T3.Azolla Int.	8.85	7.35	16.20
	-15	*-1*	*-9*
T4.30 kgN/ha	8.30	5.20	13.50
	-20	*-30*	*-25*
T5.60 kgN/ha	9.03	6.27	15.30
	-14	*-16*	*-15*
T6.Azolla Cov. +30 kgN/ha	10.49	6.91	17.40
	0	*-7*	*-3*
T7.Azolla Cov. +60 kgN/ha	10.91	8.49	19.40
	5	*14*	*8*
T8.Azolla Int. +30 kgN/ha	11.40	9.70	21.10
	9	*30*	*18*
T9.Azolla Int. +60 kgN/ha	11.98	10.02	22.00
	15	*34*	*23*

Cov. – Azolla grown as intercrop & not incorporated.
Int. – Azolla grown as intercrop & incorporated
Azolla inoculum – 3 t (fwt)/ha
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

Field Studies:

Field Experiment 1R: The wheat grown after Basmati-370 of rice Expt. 1, showed that maximum wheat straw yield was obtained for *Azolla* incorporated+FYM (T3) followed by *Azolla* incorporated (Table 19). Similarly the grain yield and straw+grain yield was significantly higher for *Azolla*+FYM and *Azolla* incorporated than the other treatments, whereas grain yield for 60 kg N/ha was significantly less than of control.

Field Experiment 2R: The wheat yield grown after 1R-6 rice experiment 3, was higher in all the treatments than control (Table 20a). As compared to control the wheat straw yield was 31-32% for *Azolla* incorporated treatments and 14-15% for urea treatments. As compared to control the wheat grain yield was 38-41% higher for *Azolla* and 16-28% for urea than control. Similarly total wheat biomass was almost double for *Azolla* as compared to urea application during rice crop.

The ^{15}N recovery of labelled *Azolla* and urea was higher in straw for urea (T6) while in grain it was higher for *Azolla* (T3), and maximum (8%) ^{15}N was recovered in rice plant from *Azolla* incorporated at 80 DAT (Table 20b). The ^{15}N recovery was higher for its application at 80 DAT than at 40 DAT.

Field Experiment 3R: The wheat harvested after growing in the plots of rice experiment 4, indicated more residual effect for *Azolla* than urea treatment (Table 21a). The wheat straw yield was higher for *Azolla* and urea applied in splits of 20+40 than 30+30 kg N/ha at 10 and 40 DAT. The grain and straw+grain yield showed a similar pattern and were maximum for *Azolla* (T2) followed by 20+40 kg N/ha of urea treatment (T6).

The total N uptake by wheat and retention in soil was generally higher for *Azolla* than other treatments (Table 21b). Higher N uptake in grain and retention in soil was found for *Azolla* and urea 20+40 kg N/ha.

The ^{15}N labelled *Azolla* and urea recovered in wheat (Table 21c) and soil after wheat was higher for urea and *Azolla* incorporated at 40 DAT than 10 DAT. Similarly the ^{15}N recovery of *Azolla* and urea in plant+soil was higher for incorporation of labelled material at 40 DAT than 10 DAT.

Field Experiment 4R: The wheat grown after rice experiment 5, showed statistically similar residual effect for *Azolla* and urea treatments (Table 22). However, slightly more grain and straw+grain yield was obtained for 45 kg N/ha and *Azolla* 2P treatment.

Field Experiment 5R: The wheat grown after rice of field experiment 6, indicated higher residual effect for *Azolla* alone or *Azolla*+urea treatments than only urea treatments (Table 23a). The wheat straw yield was upto 32% higher for *Azolla* and upto 11% for urea as compared to control. The wheat grain yield was upto 43% for *Azolla* (T9), upto 35% for *Azolla* + urea (T6) and upto 15% higher for urea (T5) than control.

The total N uptake in wheat plant (Table 23b), showed maximum amount for *Azolla* followed by *Azolla*+urea while minimum for urea alone treatment.

The ^{15}N recovery of labelled urea and *Azolla* into wheat plant and retention in soil (Table 23c) showed that ^{15}N recovered in straw was higher for urea+*Azolla* than urea or *Azolla* alone. The ^{15}N recovered in wheat grain was maximum for urea broadcasted 14 DAT (T5) followed by *Azolla* incorporated (T4) and urea+*Azolla* treatment (T3). The ^{15}N retained from *Azolla* and urea in top (0-30 cm) soil was 20-51% while in bottom zone (15-30 cm) it was 5-19%. Maximum ^{15}N (15%) was retained in top soil for *Azolla* incorporated treatment and minimum for urea treatments. Higher amounts of ^{15}N in plant+soil were found for *Azolla* incorporated treatment (T4) and urea applied at 14 DAT + *Azolla* cover and incorporated (T7), than the other treatments.

Table 19. Residual effect of Azolla and urea on wheat, grown after Basmati-370 of field Expt.1.(Field Expt.1-R)

Treatment (given to preceeding rice)	Straw (kg/ha)	Grain (kg/ha)	Straw+Grain (kg/ha)
T1.Control	3000 b	2137 b	5137 bc
	0	*0*	*0*
T2.Azolla Int.	3241 ab	2229 ab	5470 ab
	8	*4*	*6*
T3.Azolla Int.+FYM(12t/ha)	3528 a	2446 a	5974 a
	18	*14*	*16*
T4.Azolla Int.+10 KgN/ha	3021 b	2005 bc	5027 bc
	1	*-6*	*-2*
T5.60 KgN/ha	2850 b	1817 c	4667 c
	-5	*-15*	*-9*

Int.=Azolla grown as intercrop & incorporated.

Means followed by a common letter are not significantly different at the 5% level.

Figures in italics and smaller font, are % increase over control.

Table 20a. Residual effect of Azolla and urea on wheat, grown after rice cv. IR-6 of Expt.3 (Field Expt. 2-R).

Treatment (given to preceeding rice)	Straw kg/ha		Grain kg/ha		Straw+Grain kg/ha	
T1. Control	1573	b	1211	b	2784	b
	0		*0*		*0*	
T2.Azolla: 30 N15+30 N14	2077	a	1713	a	3790	a
	32		*41*		*36*	
T3.Azolla: 30 N14+30 N15	2066	a	1673	a	3739	a
	31		*38*		*34*	
T4.Urea: 30 N15+30 N14	1813	ab	1546	a	3359	ab
	15		*28*		*21*	
T5.Urea: 30 N14+30 N15	1773	ab	1409	ab	3182	ab
	13		*16*		*14*	
T6.Urea: 20 N15+40 N15	1791	ab	1481	ab	3272	ab
	14		*22*		*18*	

Azolla or urea, ordinary (N14) or labelled (N15) was incorporated at 40 and 80 DAT.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

Table 20b. Residual Azolla and urea-N recovery (%) in wheat, grown aftr rice of Expt.3 (Field Expt. 2R).

Treatment (given to preceeding rice)	Straw		Grain		Straw+Grain	
T2.Azolla: 30 N15+30 N14	0.52	c	5.17	b	5.69	b
T3.Azolla: 30 N14+30 N15	0.15	d	8.33	a	8.48	a
T4.Urea: 30 N15+30 N14	0.39	cd	2.55	c	2.94	c
T5.Urea: 30 N14+30 N15	1.23	b	4.42	b	5.65	b
T6.Urea: 20 N15+40 N15	2.13	a	3.93	bc	6.06	b

Table 21a. Residual effect of Azolla and urea on wheat, grown after rice cv.IR-6 of field Expt.4. (Field Expt.3R).

Treatment (given to preceeding rice)	Straw kg/ha		Grain kg/ha		Straw+Grain kg/ha	
T1.Control	1893	ab	1566	ab	3458	ab
	0		*0*		*0*	
T2.Azolla: 30 N15+30 N14	2177	a	1860	a	4037	a
	15		*19*		*17*	
T3.Azolla: 30 N14+30 N15	2025	ab	1639	ab	3664	ab
	7		*5*		*6*	
T4.Urea: 30 N15+30 N14	1647	b	1520	b	3168	b
	-13		*-3*		*-8*	
T5.Urea: 30 N14+30 N15	1893	ab	1567	ab	3460	ab
	0		*0*		*0*	
T6.Urea: 20 N15+40 N15	2095	ab	1694	ab	3788	ab
	11		*8*		*10*	

Azolla or urea ,ordinary (N14) or labelled (N15) was incorporated at 40 and 80 DAT.
Means followed by a common letter are not significantly different at the 5% level.

Table 21b. Residual effect of Azolla and urea on total nitrogen yield (kg/ha) in wheat, grown after rice of field Expt.4. (Field Expt.3R).

Treatment (given to preceeding rice)	Straw	Grain	Soil*	Straw +Grain	Plant +Soil
T1.Control	5.92	29.19	6.20 b	35.11	41.31 b
	0	*0*	*0*	*0*	*0*
T2.Azolla: 30 N15+30 N14	7.03	36.03	6.63 a	43.06	49.69 a
	19	*23*	*7*	*23*	*20*
T3.Azolla: 30 N14+30 N15	6.30	30.81	6.75 a	37.11	43.86 ab
	6	*6*	*9*	*6*	*6*
T4.Urea: 30 N15+30 N14	5.65	29.37	6.23 b	35.02	41.25 b
	-5	*1*	*1*	*-0*	*-0*
T5.Urea: 30 N14+30 N15	6.07	29.94	6.23 b	36.01	42.24 ab
	3	*3*	*1*	*3*	*2*
T6.Urea: 20 N15+40 N15	6.50	32.21	6.77 a	38.70	45.47 ab
	10	*10*	*9*	*10*	*10*
	ns	ns		ns	

*—soil after wheat harvest.

Azolla or urea, ordinary (N14) or labelled (N15) was incorporated at 40 and 80 DAT.

Means followed by a common letter are not significantly different at the 5% level.

Table 21c. Residual Azolla and urea-N recovery (%) in wheat, grown after rice of field Expt.4. (Field Expt.3R).

Treatment (given to preceeding rice)	Straw	Grain	Soil*	Straw +Grain	Plant +Soil
T2.Azolla: 30 N15+30 N14	1.44 b	7.30 c	0.00 d	8.74 c	8.73 c
T3.Azolla: 30 N14+30 N15	2.17 a	12.53 a	0.22 ab	14.70 a	14.93 a
T4.Urea: 30 N15+30 N14	1.75 ab	10.36 b	0.13 bc	12.11 b	12.24 b
T5.Urea: 30 N14+30 N15	1.98 a	10.82 ab	0.31 a	12.80 ab	13.11 ab
T6.Urea: 20 N15+40 N15	1.90 ab	10.27 b	0.07 cd	12.17 b	12.25 b

Same as table 21b.

Table 22. Residual effect of Azolla and urea on wheat grown after rice of field Expt. 5. (Field Expt. 4R).

Treatment	Straw kg/ha	Grain kg/ha		Straw+Grain kg/ha
T1. Control	2239	1872	ab	4111
	0	*0*		*0*
T2. Urea 15N	2386	1963	ab	4349
	7	*5*		*6*
T3. Urea 30N	2363	1914	ab	4276
	6	*2*		*4*
T4. Urea 45N	2404	2024	a	4428
	7	*8*		*8*
T5. Azolla 0P	2151	1782	ab	3932
	-4	*-5*		*-4*
T6. Azolla 2P	2323	1959	ab	4282
	4	*5*		*4*
T7. Azolla 4P	2227	1894	ab	4121
	-1	*1*		*0*
T8. Azolla 6P	2179	1718	b	3897
	-3	*-8*		*-5*
	ns			ns

Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

Table 23a. Residual effect of Azolla and urea on wheat, grown after rice of field Expt. 6. (Field Expt. 5R).

Treatment (given to preceeding rice)	Straw (kg/ha)		Grain (kg/ha)		Straw+Grain (kg/ha)
T1. Control	2522	bc	2068	b	4590
	0		*0*		*0*
T2. Urea 30N Incor. T	2513	bc	2082	b	4595
	-0		*1*		*0*
T3. Urea 30N Incor. T+ Az.Cov.T	2915	abc	2500	ab	5415
	16		*21*		*18*
T4. Az. 30 N Incor. T	2788	abc	2485	ab	5273
	11		*20*		*15*
T5. Urea 30 N BC. 2W	2726	abc	2386	ab	5112
	8		*15*		*11*
T6. Urea 30 N BC. 2W+ Az.Cov.T	3362	a	2786	ab	6148
	33		*35*		*34*
T7. Urea30NBC.2W+Az.-Cov.T+Az.Incor.4W	2554	abc	2153	ab	4707
	1		*4*		*3*
T8. Urea 60 N Incor. T	2393	c	2071	b	4464
	-5		*0*		*-3*
T9. Az.Cov. T	3332	ab	2950	a	6282
	32		*43*		*37*
					ns

T. = at rice transplanting, BC. = broadcasted, W. = week after rice transplanting.
Incor. = incorporated into mud. Cov. = Azolla grown as intercrop & not incorporated.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

Table 23b. Residual effect of Azolla and urea on the total N yield (kg/ha) of wheat grown after rice of field Expt. 6. (Field Expt. 5R).

Treatment	Straw		Grain	Straw+Grain	
T1. Control	6.75	b	34	41	b
	0		*0*	*0*	
T2. Urea 30N Incor. T	6.35	b	34	40	b
	-6		*-1*	*-2*	
T3. Urea 30N Incor. T+ Az.Cov.T	8.17	ab	41	49	ab
	21		*19*	*19*	
T4. Az. 30 N Incor. T	7.89	ab	40	48	ab
	17		*18*	*18*	
T5. Urea 30 N BC. 2W	7.43	b	40	47	ab
	10		*16*	*15*	
T6. Urea 30 N BC. 2W+ Az.Cov.T	9.67	a	44	54	ab
	43		*30*	*32*	
T7. Urea30NBC.2W+Az.-Cov.T+Az.Incor.4W	7.17	b	34	41	b
	6		*0*	*1*	
T8. Urea 60 N Incor. T	7.34	b	33	40	b
	9		*-4*	*-2*	
T9. Az.Cov. T	9.43	a	46	56	a
	40		*35*	*36*	
			ns		

T. = at rice transplanting, BC. = broadcasted, W. = week after rice transplanting.
Incor. = incorporated into mud. Cov. = Azolla grown as intercrop & not incorporated.
Means followed by a common letter are not significantly different at the 5% level.
Figures in italics and smaller font, are % increase over control.

23c. Residual Azolla and urea-N recovery (%) in wheat grown after rice of field Expt. 6. (Field Expt. 5R).

Iment	Straw		Grain		Soil after rice				Straw + Grain		Plant + Soil	
					0-15cm		15-30cm					
Urea 30N Incor. T	0.28	c	2	c	18	d	5.31	c	2	d	26	d
rea 30N Incor. T + z.Cov.T	0.35	bc	2	c	20	cd	6.06	c	3	c	29	d
Az. 30 N Incor. T	0.40	b	3	b	51	a	9.46	b	3	c	63	a
rea 30 N BC. 2W	0.60	a	4	a	29	bc	7.71	bc	5	a	42	c
rea 30 N BC. 2W + z.Cov.T	0.61	a	3	b	25	bcd	16.94	a	4	b	46	bc
rea30NBC.2W + Az.-v.T + Az.Incor.4W	0.42	b	2	c	32	b	19.28	a	3	c	54	ab
rea 60 N Incor. T	0.26	c	2	c	20	cd	6.86	bc	2	d	29	d

ice transplanting, BC. = broadcasted, W. = week after rice transplanting.
incorporated into mud. Cov. = Azolla grown as intercrop & not incorporated.
ollowed by a common letter are not significantly different at the 5% level.

DISCUSSION

AZOLLA SURVEY IN RICE AREA

Distribution and Ecology:

The presence of *Azolla* in the stagnant waters of small ponds, ditches and in slow-moving water, whereas its absence in the fast moving water (also present in adjacent area) indicated that relatively calm and quite water favoures its growth. Similarly an adverse effect of wave action and turbulence was reported on *Azolla* cultivation by Lumpkin and Plucknett (1982). Ashton (1974) observed that, after seasonal flooding most of the aggregations of *Azolla* plants were broken up and washed downstream into open water, where they were further fragmented by the abrasive wave action, and the survival rate of very small fragments was considerably reduced because of increased exposure to higher light intensity in open places. His laboratory studies indicated that increase in the shaking speed from 5 to 125 revolutions/minute resulted into decrease in plant size from 18 to 2 mm diameter. It was also observed that there was reduction of about 1/8 in relative growth rate as well as in nitrogenase activity when the plant size was reduced from 20 to 2 mm diameter. The adverse effect of disturbance on *Azolla* was also noted by Becking (1979), who found a lower rate of acetylene reduction in *Azolla* plants immediately after their transfer from one container to another one as compared to plants left undisturbed in the test vessel for 12-24 hours. These studies indicated that due to disturbance problem in fast moving water the *Azolla* could not grow there and hence it was ; . other plants especially different types of grasses were also common. Although these plants may be competing for nutrients with *Azolla* but they may have some beneficial effect on *Azolla* plants. According to Fiore and Gutbrod (1987) the presence of other aquatic plants with *Azolla* such as species of *Lemna, Pistia, Salvinia, Eichhornia, Oryza* and *Paspalum* appear to protect it from turbulence, drifting and high solar radiation, and in several regions better *Azolla* growth was also reported in the presence of paddy rice in Brazil by the farmers. According to Van Hove (1989) the wind action in open places causes premature overcrowding and thereby slows

The more frequent occurrence of *Azolla* during moderately cold months and less frequent during severe winter and almost disappearance during very hot months, in the area surveyed; indicates that the extreme temperatures do not favour its growth. The year round open air pot culture in China also showed variations in *Azolla* growth for different species, as the least growth was observed during colder part of the year having air temperature slightly less than 0 to 15°C, lesser growth at air temperature 28-31°C, while maximum growth at air temperature 20-25°C (Lumpkin and Plucknett 1982). However, in China the growth was relatively higher during summer than winter, whereas in rice area in Punjab the *Azolla* growth was better in winter than in summer. The reasonably good growth in China while disappearance of *Azolla* plants in Punjab during summer, may be due to relatively less severe summer in China having maximum air temperature 28-31°C whereas in Punjab maximum temperature may rise to about 45 or even 47°C as already shown in Fig. 5. Secondly the better growth in Punjab during severe winter may be due to less severe temperature during winter in Punjab than in China, where the minimum temperature was sometimes below 0°C. The biomass production by 4 strains of *Azolla* in Philippines also showed variation during the year round cultivation and maximum growth was obtained from January to March and again in September and October when the average air temperature was around 25°C, while minimum growth was found in April when the temperature rose to about 35°C (Mabbayad 1987). According to Satpathy and Singh (1992) the productivity of *Azolla* depends on seasonal variation during the year. He observed maximum growth and nitrogen fixation in October when the water temperature was around 30°C. In a 2 years study, they found maximum fresh biomass and nitrogen fixation during September and October (water temperature 20-25°C) which decreased in the coming months and became minimum during April-June when water temperature rose to above 25°C (Singh 1992). Thus the pattern of *Azolla* growth, observed in rice area of the Punjab followed almost similar pattern as reported by Singh (1992), which may be due to similar climatic conditions of Pakistan and India.

composition of water standing on soil after equilibrium will depend on the type of water reaching there and the inherent properties of submerged soil. Thus floodwater properties and its chemical composition are very important for its growth and nitrogen fixation.

The chemical analysis of water supporting *Azolla* growth, as mentioned under Results, indicated that it can grow in a wide range of nutrient concentrations. Similarly the ionic strengths of various nutrient solutions also showed a considerable variation in different culture media used for *Azolla* (Becking 1979). He has shown that, in 11 culture media used for *Azolla* cultivation, P concentration (expressed as H_2PO_4 ion) varied 0.5-6, Na^+ 0-2.2, K^+ 1.3-10, Ca^{++} 1.4-12, Mg^{++} 1.6-18.8, SO_4^{--} 0.4-24 and Cl^- 0-12.8 meq/L. So he concluded that *Azolla* can be cultivated in a simple N-free medium usually containing only a few essential salts such as magnesium sulphate, calcium chloride, potassium chloride, potassium sulphate and an iron source.

To find the relationship of different floodwater properties to *Azolla* growth, the enveloping graph technique of Blandreu and Ducerf (1980) was used and it is the most suitable technique for field data in which more than one factors are acting simultaneously. For example, the Fig. 7e shows that at the same concentration of bicarbonates i.e. 3 meq/L, the *Azolla* cover was about 10, 50, 90 and 100% for different water samples. The enveloping graph showed that optimum concentration is 3 meq/L, indicating that as soon as the bicarbonate concentration reaches to 3 meq/L, maximum growth becomes possible, as it was observed for one of the water sample, and the lower growth at the same concentration for other samples is due to some other factors limiting *Azolla* growth in these waters. Thus the enveloping graph technique is ideal for finding relationships between different factors and threshold or optimum concentration for a nutrient under field conditions.

The lower *Azolla* growth, indicated by its cover on floodwater surface, at higher floodwater pH (>8.7) as indicated in Fig. 7a, may be due to lower availability of certain nutrients. At alkaline pH, the availability of some macronutrients like Ca, and Mg is said to be reduced due to their decreased solubility. Similarly the availability of micronutrients like Fe, Mn, Zn, Cu and B (except Mo) also decreases at higher pH. The availability of P

culture media buffered at pH 5,6,7 or 8 but the growth declined at pH 9. It was also observed that concentration of iron in *Azolla* was about 3 times greater in plants grown at pH 6 than at pH 9 (Peters and Calvert 1982), indicating lower availability of Fe at higher pH.

The growth of *Azolla* decreased at higher electrical conductivity (EC) as shown in Fig. 7b, which may be due to adverse effect of higher salts leading to osmotic problems. It was reported that *Azolla* plants wilted at 1.5-1.9 g salts/L, whereas in lake water having 0.16-0.38 g salts/L its growth was normal (Becking 1979). A decrease in growth and nitrogenase activity of *Azolla pinnata* with an increase in EC of culture medium from 0.75-5.0 dS/m was observed in green house conditions by Ali et al (1990). Similarly, a decrease in nitrogen fixation, chlorophyll content, photosynthesis and respiration was observed at 0.32% sodium chloride in four species of *Azolla* by Kannaiyan (1992).

Since less than 0.1 ppm P in floodwater causes P deficiency in *Azolla* (Ali and Watanabe 1986), therefore, the low concentration of P in most of the samples may be a factor leading to lesser growth at most of the sites. As explained above the high pH tends to precipitate phosphorus with calcium. Secondly, H_2PO_4 ions which are more available than HPO_4 ions, are predominant at slightly acidic to neutral pH i.e. 4.5-7.5 (Brady 1984). So the low concentration of floodwater p in our samples may be due to the alkaline pH of most of these waters.

Good growth of *Azolla* was observed in a wide range of Ca+Mg concentration of floodwater, and required concentrations of these elements were present in most of the water samples (Fig. 7c). The critical concentration for Ca and Mg was reported to be 0.4 and 0.5 mmol/L by Kitoh and Shiomi (1991). Thus the floodwater of rice area had more concentration of Ca+Mg than the threshold level which may be due to calcareous nature of the rice soils of the Punjab (Chaudhry 1978).

The lower *Azolla* cover at higher concentration of carbonates (Fig. 7d) while better growth at zero to lower concentrations indicates that presence of carbonates in floodwater is not essential for its growth. The carbonates especially of Na and K tend to increase the

A moderate amount of bicarbonate favoured *Azolla* growth (Fig. 7e). The bicarbonates act as buffer for stabilization of pH and secondly their dissociation releases CO_2 which may be captured by *Azolla* for its photosynthesis. It was also reported that uptake of K from $KHCO_3$ was about 7 times higher than KCl, due to the specific action of HCO_3 on opening of guard cells (Leopold and Kriedemann 1975). Thus a moderate amount of this ion in the floodwater helped in better growth of *Azolla*.

The lower concentrations of chlorides and sulphates (Fig. 7f and g) was useful for *Azolla* growth, while the higher concentrations did not favour its growth. The higher concentration of sulphates are reported to reduce the availability of divalent ions like Ca^{+2} and Mg^{+2} forming insoluble precipitates, while higher concentrations of chloride exert their specific toxic effect on plants (Brady 1984), and higher concentrations of chlorides may also reduce the uptake of some cations like K^+ as explained above (Leopold and Kriedemann 1975).

The wider range of organic matter in floodwater was acceptable for *Azolla* growth (Fig. 7h) and a reasonable concentration of Fe (0.1-0.2 ppm) and Cu (traces to 0.01 ppm) as shown in Fig. 7i and 7j, were helpful for *Azolla* growth. The concentration of Fe in water depends on pH of the solution and for *Azolla* growth it is considered crucial (next to P) for occurrence of *Azolla* in natural waters (Becking 1979). Higher concentration of Fe was required at pH 7.5 than at pH 4.5-6.5 to obtain good growth of *Azolla* (Watanabe et al 1977). Only 1/9 of the biomass was obtained in Fe free as compared to Fe containing culture medium and ferrous ions were more available than ferric ion, and ferrous ion or iron as iron-humus complex was found necessary for *Azolla* growth at neutral (7.0) or higher (8.1) pH (Olsen 1970). He has reported that *Azolla* growth was better in brownish water having organic matter, and it was also found that there was 200 times more iron in the solution which was obtained from black mud (due to sulfides of Fe and Mn) having reduced form of iron as ferrous sulfide. According to Becking (1979), the chelated iron as iron-humus is useful for iron availability in natural waters, while chelation with citrate or EDTA (iron-versenate) is used in synthetic culture media.

The higher amount of Na is said to be toxic for plant growth in saline or sodic soils (Brady 1984).

On the basis of analysis of floodwater and relationships of its properties to *Azolla* cover, it can be summarized that for better growth of *Azolla* under field conditions, only one parameter is not sufficient, but a combination of different properties is required for its optimum growth. Thus to have a better growth of *Azolla*, in addition to optimum concentration of P (>0.15 ppm), desirable values of pH, EC, cations, anions, organic-C and micronutrients as given in Table 24, are also required. Some of these value may appear less than the threshold values given by different workers for limited culture conditions. But in field conditions a lower concentration may work, as a continuous supply of nutrients is mostly assured from the soil. It was reported that *Azolla* growth declined in laboratory batch culture by 30th day, whereas the same water with the same initial P concentration in channel water *in situ* continued supporting good *Azolla* growth and produced more than 30 times *Azolla* biomass than batch culture in 50 days (Costa et al 1994).

The interaction of different nutrients may affect their availability, as higher amount of P depresses the availability of Fe and Zn, while higher concentration of bicarbonates reduces Fe availability to plants (Brady 1984). The poisoning of iron to *Azolla* when calcium is less than 2 ppm or so, was reported by Olsen (1970). In addition to P some other factors were thought to be operating for the formation of bloom in the river, as P concentration was same during both the years, but *Azolla* bloom was formed only during one year (Carrapico 1994). This observation also suggests that positive interaction of more than one factors is required for *Azolla* growth. According to Lumpkin (1987a) a true picture of environmental requirement of *Azolla* can not be given by describing individual requirement in isolation, as the interaction or indirect effects of factors are often more important than direct effect. Thus based on our observations and reports of other researches, it can be concluded that in addition to suitable climate, presence of all the essential nutrients in an optimum balance are required for better growth of *Azolla* under field conditions.

Table 24. Summary of relationships of floodwater properties and nutrient concentration on Azolla growth.

Floodwater property or nutrient or ratio	Value or concentration or ratio	Azolla cover observed mostly
pH	< 8.7	High
	> 8.7	Low to medium
EC (dS/m)	0.3 -1.1	High
	> 1.1	Low to medium
Ca + Mg (meq/L)	1.5 - 3.5	High
	> 3.5	Medium
Carbonates (meq/L)	0 - 1	High
	> 1	Low to medium
Bicarbonates (meq/L)	2 - 5	High
	> 5	Medium
Chlorides (meq/L)	1 - 4	High
	> 4	Low to medium
Sulphates (meq/L)	1 - 4	High
	> 4	Low to medium
Organic Carbon (ppm)	Traces - 100	High
	> 100	Medium
Iron (ppm)	0.5 - 0.2	High
	> 0.2	Medium
Copper (ppm)	Traces - 0.01	High
Na / Ca	< 10	High
	> 10	Low to medium
Na / K	7 - 25	High
	< 7 or > 25	Low to medium

High = 75 % area of floodwater covered with Azolla though at some places
Medium = Approx. 25 - 50 % area covered with Azolla
Low = 25 % area covered with Azolla

TEMPERATURE STUDIES OF *AZOLLA*

Selection of Heat Tolerant Strain:

Since rice is grown during summer and the native *Azolla* found in rice area was sensitive to high temperature, therefore, selection of a heat tolerant *Azolla* strain capable of growing during rice season was a prerequisite for its use in rice based cropping system.

The adaptation and selection of heat-tolerant *Azolla* was done by shading the plants and cooling the water during severe summer at noon, as gradual increase in temperature is more tolerable than sudden changes in environmental conditions. Tung and Watanabe (1983) observed that growth rate of *Azolla* strains did not stabilize immediately after temperature change but instead required at least 3 week's acclimation for strains that could adapt to increased temperature. It was reported that during a short period of high temperature, nitrogenase enzyme is especially active if *Azolla* has been previously grown in warm (30/20°C), instead of cool (20/10°C) or cold (15/5°C) environment (Talley and Rains 1980). The addition of farmyard manure and phosphorus as superphosphate into soil-water culture appeared to help *Azolla* survival during severe summer. According to ZAAS (1975) all decomposed farmyard manures contain various organic and inorganic nutrients as well as plant growth promoting substances, and despite their low nitrogen content, they have a positive effect on *Azolla* growth and especially on the revitalization of weak plants. Thus gradual exposure of *Azolla* to increasing temperature of summer and supplying the essential nutrients helped in adaptation and selection of heat tolerant strain, which was used in all the laboratory and greenhouse studies related to its use as a biofertilizer in rice-wheat cropping system.

The identification of *Azolla* as *A. pinnata* made on the basis of most of the vegetative characters and some sporocarp features was a little bit doubtful, therefore, Dr. Watanabe was requested to confirm identification as facilities were available at IRRI. He, on the basis of detailed study of vegetative and reproductive structures (like sporoderm), not only confirmed our identification but identified further to varietal level (*A. pinnata* var. *imbricata*) which was not possible in our Institute or in Pakistan. Although the occurrence of *Azolla*

taxonomist. Thus the identification of local *Azolla* to varietal level may be a new information about *Azolla* in Pakistani flora. The occurrence of *A. pinnata* has also been reported from India by Singh (1979), Nepal by Joshy (1983), while in China and other countries by Lumpkin and Plucknett (1982).

Effect of Temperature on Growth and Sporulation:

The use of *Azolla* in the tropics is sometimes restricted due to its low tolerance to high temperature (Lumpkin and Plucknett 1982, 1987a, Watanabe 1994). Therefore a greenhouse study was undertaken to study the effect of water temperature on *Azolla* growth, and sporulation which is considered very important for its economical use in rice ecosystem as already mentioned under constraints. The greenhouse study showed that maximum growth of *Azolla* occurred at water temperature of 30°C as compared to 15, 20, 25 and 36°C (Fig. 7). At IRRI, Watanabe et al (1981) tested 27 strains of *Azolla* at 3 temperature regimes having 8°C difference in day/night temperature i.e. 26/18°C (average 22°C), 33/25°C (average 29°C) and 37/29°C (average 33°C). They found that relative growth rate was higher at average temperature of 33°C than at 22°C, and maximum value was observed for some strains of *A. pinnata* as compared to other species/strains. The maximum biomass production in our case was at 30°C, the 2°C difference may be due to difference in strain as different strains differ in temperature tolerance or due to difference in experimental conditions as we grew *Azolla* at constant temperatures whereas they tested at 8°C difference between day and night temperature. Talley and Rains (1980) observed that exponential growth rate of *A. filiculoides* increased linearly with temperature between 10/1 and 25/15°C and remained high up to 35/25°C (average 30°C), and no growth at 40/30°C (average 35°C), thus our results agree with their results as we also found increase in biomass production up to 30°C while decrease at 36°C.

The high temperature is reported to adversely affect the nitrogenase activity in *Azolla*. Becking (1979) found that nitrogenase activity measured by ARA reached to its maximum value at 30°C in *A. pinnata* and decreased at higher temperature especially if over 37°C.

decrease in protein as well as chlorophyll contents were observed due to senescence in some of the *Azolla* species (Tung and Watanabe 1983, Kannaiyan 1992), but such changes were not so apparent in heat tolerant strains of *A. pinnata* (Tung and Watanabe 1983).

In fields, sometimes *Azolla* may be growing in limited-nutrient conditions at different temperatures prevailing during different seasons. To know the effect of temperature on Azolla growth in such situations it was also grown in relatively nutrient-exhausted culture medium. The *Azolla* biomass production under stressed conditions i.e. in exhausted culture solution was low, which may be due to lower amount of nutrient in this condition. Costa et al (1994) found that in the limited volume of channel water, P decreased to one-third of initial concentration by 10th day of *Azolla* cultivation, and thereby its growth decreased significantly by 30th day of incubation. Tung and Watanabe (1983) found that ARA values of *Azolla* grown in P-deficient culture solution were more adversely affected by high than at lower temperature and heterocyst frequency in *Anabaena* was also reduced at higher temperature and was slightly more prominent in P-free cultures.

The pigments of *Azolla* were reported to be affected by seasonal temperature in India (Satpathy and Singh 1992). They observed lower amounts of chlorophyll during severe summer (May-June) and winter (December-January); whereas maximum anthocyanin content, giving the plants a reddish colour, was observed during the month of May and minimum during October. They also reported that a positive correlation existed between chlorophyll content and nitrogen fixation irrespective of the P dose applied or strain used or the season. Similarly a reduction in chlorophyll was also observed at higher temperature of 37/29°C than at 26/18°C, whereas anthocyanin pigment was higher in P-free medium and at low temperature treatment (Tung and Watanabe 1983), and this may be the reason of reddish plants at 15 and 20°C as compared to plants grown at 25 and 30°C in our experiment. In our experiment smaller and reddish plants were present at 36°C. The smaller size of the plants may be due to senescence at higher temperature leading to smaller fronds. The reddish colour may be due to higher anthocyanin pigmentation, as at higher temperature more of this pigment was formed in *Azolla* as mentioned above. According to Satpathy and

The sporocarp formation is said to be affected by temperature and spore formation was 17-85% in the field conditions during September to March when the average air temperature was around 27°C and growth was also maximum (Mabbayad 1987). Similarly formation of spores was observed during March-April in Portugal when *Azolla* density was also maximum (Costa et al 1994). In our experiment sporocarps were formed at 25 and 30°C, thus our results are in quite agreement to above observation of Philippino scientists. It was reported that time needed for sporocarp formation was lesser (15 days) for higher temperature (32°C) as compared to longer time (45 days) at lower temperature around 20°C for *A. filiculoides* (Talley and Rains 1980). At IRRI, the temperature of 26/18°C led to maximum vegetative growth and higher number of sporocarps in *A. mexicana* and the ratio between micro and megasporocarps was also affected by temperature (Watanabe et al 1981).

AZOLLA CULTIVATION

Azolla Cultivation in Defined Culture Media:

Biomass, chlorophyll content and ARA: Although various culture media have been reported for growing *Azolla* (Becking 1979, Kitoh and Shiomi 1991, Watanabe et al 1977, Yatazawa et al 1980) but plants maintained in culture media different to field conditions, may become adapted to such culture media and hence may give some problem in cultivation when inoculated under different conditions in the field. To minimize such adaptation during cultivation, different culture media were synthesized according to the composition of floodwater having good *Azolla* growth in rice area of the Punjab.

The comparison of different culture media showed that IRRI medium adjusted to pH 6.5 was the best (Fig. 9a) which may be due to higher availability of the nutrients at the slightly acidic pH . Based on the relations between pH, microbial activity and nutrient availability, it was summarized that, on the whole, a pH of 6-7 promotes the availability of most of the essential macronutrients like Ca, Mg, N, P, K, S and also the micronutrients like Fe, Mn, Zn, Cu, Co, Mo and B. It was concluded that if pH is suitably adjusted for phosphorus, the other plant nutrients, if present in adequate amount, will be satisfactorily

pH 2-7 the maximum available type i.e. $H_2PO_4^-$ ion is dominant and at higher pH HPO_4^{--} and PO_4^{---} ions become dominant. Thus the pH 6.5 of IRRI medium of Watanabe et al (1977) helped in the availability of P and other nutrients and led to maximum biomass production and chlorophyll content of *Azolla* (Fig. 9a).

When the pH of the IRRI culture medium was adjusted to 8.0 the situation changed and growth as well as chlorophyll content were reduced and were lower than KB culture medium (Fig. 9a). The reason for poor performance of this medium at pH 8 may be due to the lower availability of nutrients and interaction of some nutrients at alkaline pH. As mentioned above, at high pH, the HPO_4^{--} and PO_4^{---} dominate and they tend to react with calcium at alkaline pH to form insoluble compounds like $CaHPO_4.2H_2O$ and $Ca_3(PO_4)_2$ (Brady 1984). It was also observed that when the pH of this medium was adjusted from original pH to 8.0, precipitation in the medium occurred and it became turbid. Since this medium contains higher amount of phosphorus, therefore P might have caused precipitation of most of the Ca and of the micronutrients like Fe and Zn in the solution (Brady 1984), therefore, *Azolla* growth was reduced at pH 8 as compared to its growth obtained at pH 6.5.

The better performance of KB medium compared with other culture media may be due to lower contents of its bicarbonates and sulphates. The higher amounts of sulphates was found to be undesirable for *Azolla* growth (Fig. 7g) and higher amounts of bicarbonates are reported to interfere with iron metabolism and may lead to chlorosis (Brady 1984).

The acetylene reduction assay (ARA) for measurement of reduction of acetylene into ethylene gives a good index of nitrogenase activity (Li et al 1987), therefore, ARA was performed for different culture media. The ARA of *Azolla* was expressed on chlorophyll content basis instead of on biomass basis, as it gives a better indication of life activity of living tissue than the fresh or dry weight which may include some senescenced dead parts of the plant. Like higher biomass production and chlorophyll content in IRRI medium at pH 6.5, the ARA values were also higher than the other culture media as well as IRRI medium at pH 8, which may be due to better availability of nutrients at pH 6.5, as explained above. Since the better availability of nutrients helps in better physiological activity therefore nitrogenase activit

activity, as a very low ARA value was observed for *Azolla* grown in P-free than in culture medium containing P source (Watanabe et al 1977).

In general, the rate of ethylene production, decreased with increase in incubation period (Fig. 9b), which may be due to increase in temperature with time or some other changes in the micro-environment of the incubation tube. It was reported that the rate of ethylene production was found to change with change in environmental conditions like light and temperature, and ARA value for 4 *Azolla* species reached to the peak value by 10th minute of incubation period and declined significantly after one hour of incubation (Li et al 1987). Secondly the nitrogenase enzyme obtains ATP for reduction of the substrate from the photosynthetic products (Peters et al 1980, Ladha and Watanabe 1987), therefore, limited supply of photosynthates during night may have caused lower rate of ARA for 23 hours incubation period. It was reported that dark-aerobic ARA seldom exceeds 40% of the light-aerobic ARA and declines as endogenous sources of reductant are depleted (Peters et al 1981, Becking 1979).

Effect of Humic Acid on Growth and N_2 Fixation: Farmyard manure is commonly used to enhance *Azolla* growth in China (ZAAS 1975) and Vietnam (Lumpkin and Plucknett 1982) and major portion (65-75%) of organic matter present in most soils and waters consists of humic substances (Schnitzer 1982). Therefore effect of humic acid, the major component of humic substance, was studied on *Azolla* growth and its nitrogenase activity. It was observed that addition of humic acid (HA) increased the biomass production and enhanced the nitrogenase activity of *Azolla* (Fig. 10). Because humic substances contain ionizable hydrogen in aromatic, aliphatic and in phenolic groups (Schnitzer 1982), therefore, they can sorb charged metal cations, organic and inorganic molecules having some charge on them and also neutral organic molecules by hydrophobic adsorption rather than phase partioning (Murphy and Zachara 1995). Thus the addition of HA in the *Azolla* culture medium may have reduce the precipitation of useful metals by chelation and so helped in a regular supply of nutrients to *Azolla*. The presence of iron-humus complex was found to be necessary for *Azolla* growth in the Danish lake water by Olsen (1970). In addition to

N and 0-2% S (Schnitzer 1982) therefore they are also said to supply the nutrients or taken up directly by the plant roots (Schnitzer 1978). It was reported that soaking of wheat seeds in humic acid solution resulted into increased growth of seedling and better development of seedling roots, and the adverse effect of salinity was also reduced (Azam and Malik 1983). Since very small concentrations of HA were used in the *Azolla* culture medium, therefore, its direct role of supplying nutrient was least possible, however, its role as auxin like substances and regulation of supply of essential substances, may be more important for enhancing biomass production and nitrogenase activity in *Azolla*.

Azolla Cultivation in Undefined Culture Media:

The defined culture media are really nice for laboratory or greenhouse cultivation on small scale, but they are expensive and practically impossible to be used by the farmers for maintenance of *Azolla* culture in their nurseries in field conditions. Therefore for maintaining the *Azolla* culture by the farmers, simple and undefined culture media were tried and compared with defined culture media for *Azolla* growth and nitrogen fixation efficiency.

Preliminary Experiment: The preliminary experiment using different soils, waters and cow dung showed that pond water containing pond soil and cow dung was better for *Azolla* growth (Fig. 11). The higher growth for this treatment seems due to different factors. Since cattle dung contains 0.3-0.4% N, 0.10-0.15% P_2O_5 and 0.15-0.2% K_2O (Gaur 1992), therefore it may supply essential nutrients to *Azolla* and hence improving its growth. In addition to supplying nutrients, the organic acid in the dung may help in chelating the nutrients and thereby improving supply for better *Azolla* growth. The pond water and pond soil are product of reduced conditions of soil. Olsen (1970) has reported that brownish lake water containing high amount of Fe led to abundant growth of *Azolla*, and black mud containing ferrous sulphide formed by the anaerobic process liberated 200 times more iron in the ferrous form and caused better growth of *Azolla*. He also observed that *Azolla* did not grow well in solution of pH 7 when there were only ferric ions as iron source, and the plants soon became chlorotic. Kulasooriya et al (1994) observed that in sand-water culture pot

cow dung < peat < poultry manure, although each source was added to supply 1g P/m^2. It was also found that *Azolla*-N accumulated per pot was up to 100% higher for cow dung than superphosphate. Thus the cow dung and black soil of pond led to higher growth of *Azolla* due to better supply of nutrients and particularly of P and iron to *Azolla*.

Comparison of Defined and Undefined Culture Media: The comparison of defined and undefined culture media showed that *Azolla* growth and its rate of nitrogen fixation as estimated by ARA, was better in soil-water culture containing 5% soil and 0.5% farmyard manure, compared with water culture medium of KB, IRRI and FYM (Fig. 12). The better growth and nitrogen fixation in soil+FYM culture medium may be due to better supply of nutrients from two sources i.e. soil and FYM, and stabilization of pH due to larger buffering capacity of the soil. The farmyard manure is reported to have 0.5-1.0% N, 0.15-0.20% P_2O_5, 0.5-0.6% K_2O (Guar 1992), while sufficient amounts of other nutrients like Ca 2.4-7.4, Mg 1.6-5.8, S 1.0-6.2, Fe 0.08-0.93, Zn 0.03-0.18, B 0.02-0.12, Mn 0.01-0.18, Cu 0.01-0.03 and Mo 0.001-0.011 kg/metric ton (g/kg or mg/g) are also present in FYM (Brady 1984). Thus FYM is a good source of balanced nutrients, and organic acid in FYM may further help in chelation of nutrients for a better and regular supply of nutrients leading to higher growth and nitrogen fixation in *Azolla*.

Long Period Cultivation in Soil-Water Culture: *Azolla* has to be maintained in the nursery throughout the year therefore long-period growth of *Azolla* in soil-water culture medium was studied. The more biomass production for FYM, IRRI medium and other treatment than control (soil+water) as shown in Fig. 13, indicated that nutrient supplement are needed into soil-water culture for better *Azolla* growth. Similarly in a greenhouse experiment in Egypt the *Azolla* biomass production for *A. pinnata* and *A. filiculoides* was higher for Yoshida's culture solution, of rice followed by peat (10g/850 ml tap water) than in soil-water (25 g sterilized soil + 750 ml tap water) culture (Abd-Alla et al 1994).

The results showed that higher cumulative amounts of *Azolla* biomass were produced for addition of Fe+FYM, P or Fe or FYM into soil water culture than the other treatments (Fig. 13) indicating that P and Fe were the major nutrients to be supplemented for better

major limiting nutrient. Similarly slightly more *Azolla* growth for only P than IRRI medium and only FYM (containing all nutrients) indicated that among macronutrients P was the major requirement.

The higher biomass for only FYM than P+FYM and for Fe+FYM than Fe+P+FYM indicated that P in FYM was sufficient for *Azolla* growth, and the application of additional P depressed *Azolla* growth which may be due to interaction of P with Fe or other micronutrients, as application of large quantities of phosphate fertilizer reduces the uptake of iron, zinc and some of the other micronutrients (Brady 1984).

During the initial (first) period FYM application gave lesser yield than Fe, micronutrients and IRRI medium, but later on yield in FYM surpassed these treatments. The slower action of FYM may be due to requirement of some period for its decomposition and release of nutrients. Similarly a much higher *Azolla* yield was obtained in second harvest than first harvest for cow dung than P fertilizer by Kulasooriya et al (1994). Both these studies indicate that organic manures are very useful for sustaining *Azolla* growth for a longer period of cultivation.

The much higher *Azolla* biomass production for Fe+FYM compared with FYM or Fe alone suggests that there was some positive interaction between them. It appears that, Fe applied as ferric chloride was less available, and the reduced condition in soil due to anaerobic decomposition of FYM led to reduction of ferric into ferrous ions (Ponnamperuma 1977) and thus increased its availability, and the ferrous ions liberated by black reduced mud were reported to be more available to *Azolla* (Olsen 1970). It is also possible that chelation of Fe ions by organic acids, produced from decomposition of FYM, also helped in its availability and improved *Azolla* growth.

The morphology of *Azolla* plants after 10 months of cultivation indicated that *Azolla* plants were healthier (dark green, large size, thicker roots) and more in number in pots having FYM, FYM+Fe and FYM+Fe+P compared with the remaining pots indicating that application of FYM along with Fe or P is useful for long-period maintenance of *Azolla* culture in out door conditions.

experimental plots (as required for planned treatments) without bringing it from outside the test plots, so only 1.5 kg *Azolla*/m^2 was incorporated, and thus growing *Azolla* of desired biomass in given time and space was a major constraint for carrying out the trials in 8 different countries (Watanabe 1987).

The possibility of nutritional constraints like P, Zn and Cu in saline soils (Lumpkin 1987a) and lower availability of some micronutrients like Fe (Brady 1984) and Zn deficiency in flooded soils of Pakistan (Ponnamperuma 1977) has been reported. Therefore, to diagnose the P, Zn and Fe constraint for *Azolla* growth, a local marginally saline soil was used, as rice is usually grown in such soil. It was found that P was a major limiting nutrient for *Azolla* growth in this soil (Fig. 13). The nutrient missing technique (Watanabe et al 1977) is useful for diagnosis of most limiting nutrient as minimum biomass is produced in the absence of that nutrient which is the most limiting one as compared to the treatment containing all the nutrients. Thus the minimum *Azolla* biomass and ARA value in -P (+Zn+Fe) indicated that P was a major limiting nutrient and the addition of Zn and Fe did not improve its growth significantly. The second lowest biomass of *Azolla* was produced in -Fe (+P+Zn) but biomass produced was higher than -P thus Fe was another limiting nutrient limiting *Azolla* growth in this soil. Since as compared to complete nutrient treatment (+P+Zn+Fe), there was about 50% *Azolla* biomass and 20% ARA value for -P, while around 80% biomass and 95% ARA value for -Fe, therefore P was more important limiting nutrient than Fe for *Azolla* in the tested soil. The problem of P deficiency has been reported to be the most common for *Azolla* growth in different countries (Ali and Watanabe 1986, Lumpkin 1987a,b, Singh 1979, 1992, Watanabe 1994), while Fe was also found to be a problem due to high pH at some places (Olsen 1970, Rains and Talley 1979, Lumpkin 1987a).

Since P was found to be the major problem in the above said experiment, therefore finding its optimum dose for *Azolla* growth was the next step, as this was not possible with missing technique. To find the optimum dose of P for *Azolla* growth, the same soil was used, and it was found that a lower dose of P (10 kg P_2O_5/ha) was better than higher doses

quantities of P fertilizer can adversely affect the supply of some micronutrients and uptake of both Fe and Zn may be reduced due to presence of excess phosphate. The formation of less soluble forms of both P and Zn like Zinc ammonium phosphate and zinc phosphate, causing reduced availability of these elements to plants has also been reported (Ponnamperuma 1977).

The split application of P i.e. 10 kg P_2O_5/ha in 4 splits (2.5 kg/week) was slightly higher than application of the same amount at one time, and the split application of P was also reported to be useful for better growth of *Azolla*, and its application at the rate of 1 kg P_2O_5/ha every 4 days was recommended by Watanabe (1982), while 4.6 kg P_2O_5/ha every week was recommended for Indian soils by Singh (1979). The better response of split P than the single application may be due to maintenance of higher level of P in floodwater, as P level was found to decline to its equilibrium level within 7 days of its application into floodwater (Ali and Watanabe 1986).

PEST MANAGEMENT

Pests and their Control:

During cultivation of *Azolla* in the nursery or experimental plots, the regular pest for *Azolla* were water snails, while seasonal attack was by Nymphula sp. (Lepidoptera). The water snails, which were observed in local conditions, have also been reported eating *Azolla* in other countries (Fiore and Gutbrod 1987, Lumpkin and Plucknett 1982, Watanabe 1982, ZAAS, 1975) while the seasonal attack of larvae of Lepidoptera insects was reported from China (ZAAS 1975, Zhang et al 1987), India (Singh 1992), Philippines (Mochida et al 1987) and other countries (Lumpkin and Plucknett 1982). The high humidity coupled with warm temperature is reported to encourage insect multiplication (Lumpkin 1987a, Watanabe 1982). As both of these requirements were not commonly fulfilled in local conditions due to dry climate prevailing during most of the months (Fig. 5), therefore seasonal and a lesser insect attack was observed as compared to tropical countries having hot and humid conditions. The problem of pests was also found of less serious nature in relatively dry climate of Africa

may be due to difference in type of algal flora or soil or climate conditions. Similarly fungi were not a big problem and fungal growth was observed on the *Azolla* plants damaged by insects or after the death of plants due to some ecophysiological stress. The lower fungal attack may be due to dry conditions prevailing most of the period of the year.

For control of *Azolla* pests, various pesticides has been used (Lumpkin and Plucknett 1982, Fiore and Gutbrod 1987, Singh 1992, Zhang et al 1987). However, pesticides used by farmers and easily available in the local market were used for controlling insects in *Azolla* nursery and field cultures. The Akar and Endrin were more toxic than the other 3 pesticides (Fig. 15a) which may be due to their specific effect on tested water snails, and Endrin was reported to be neurotoxic to pests (Hill 1975). Similarly various mortality values (57.2-99.5%) for grey-snout moth were observed for six insecticides and one bioinsectide in China (Zhang et al 1987).

Effect of Pesticides on *Azolla* Growth:

The adverse effect of pesticides on *Azolla* indicated that it was higher for Endrin and Akar than the other 3 pesticides (Fig. 15b) Nuvacron (Monocrotophos) was toxic to *Azolla* and gave 73% *Azolla* biomass that of control. The use of this insecticide (0.3 L/ha) by Fiore and Gutbrod (1987)was reported to control the pests efficiently in Brazil but its effect on *Azolla* growth was not mentioned.

The use of Furadan (carbuforan) has been recommended by different workers for pest control of *Azolla* (Lumpkin and Plucknett 1982, Satpathy and Singh 1992, Watanabe 1982). In our studies it was found that Furadan was least toxic to *Azolla* (Fig. 15b) and was used for pest control in all the laboratory and field studies. Although it was least toxic but it still gave *Azolla* yield which was 91% that of control (Fig. 15b). Kannaiyan (1992) observed more stimulatory effect of carbofuran at lower concentration (2.5 ppm) compared with that at higher concentration (5 ppm) for relative growth rate, and also on nitrogenase activity and chlorophyll content of two strains of *A. pinnata*, while in other strain of the same species its effect was depressive especially at the higher concentration. The higher depressive effect

The addition of 5 ppm carbofuran into culture medium stimulated ammonia excretion by different nitrogen fixing blue-green algae (including *Anabaena Azollae*), immobilized in polyurethane foam. The increase in ammonia liberation was attributed to the inhibition of glutamine synthetase activity in cyanobacteria, leading to more excretion of ammonia into culture medium (Umma and Kannaiyan 1991). From this study it can be inferred that since *Azolla* is in direct contact with the insecticide therefore the said enzyme, responsible for NH_3 assimilation (Ladha and Watanabe 1987), should also be partially inactivated. Thus the assimilation of ammonia excreted by the endophyte *Anabaena* will be reduced by *Azolla* leading to a lesser growth especially at higher concentration of carbofuran.

NITROGEN FIXATION IN *AZOLLA*

Although total gain of nitrogen in nitrogen free culture medium or ARA gives a good estimate of nitrogen fixation by *Azolla*, as already performed in cultivation of *Azolla* in defined culture media. However, in natural conditions of rice fields there is no nitrogen-free floodwater, as nitrogen from soil or fertilizer or from both the sources will be present in varying amounts in different situations. The nitrogen fixation, by *Azolla*, in the presence of combined-N is likely to be different from that in the N-free culture medium. Therefore, to see the effect of combined-N on *Azolla* growth and nitrogen fixation, greenhouse and field experiments were conducted.

Nitrogen Fixation in the Presence of Combined N:

In the greenhouse study it was found that as compared to control (N free KB medium) the lower concentration of ammonium-N (14 ppm) enhanced *Azolla* growth while at higher concentrations (28 and 42 ppm) its growth was retarded, but it was still slightly higher than control(Fig. 16). It was reported that the effect of combined N in the form of ammonium sulphate was variable on different *Azolla* species as with an increase in concentration from 0 to 20 ppm, the dry biomass increased for *A. caroliniana*, remained unchanged for *A. filiculoides* whereas decreased in *A. microphylla* and *A. pinnata*

for NH4-N it increased up to 2.5 mM and then decreased afterwards, while it decreased from 1.25 mM to all the remaining concentrations (5, 10, 15 and 20 mM). Ito and Watanabe (1983) observed less inhibitory effect of nitrate than ammonium N, on nitrogen fixation due to preferential distribution of the former type in the root and older parts (having less ARA activity) in *Azolla*. These results show that inhibitory effect of combined nitrogen depends upon the strain of *Azolla* as well as on the type of nitrogen present in the culture medium. Reviewing the results of other workers and their own work, Kitoh and Shiomi (1991) have concluded that lower concentration of NH_4 and NO_3, whereas higher concentrations of urea i.e. up to 10 mM are fully suitable for *Azolla* growth. However, their data showed that as compared to growth at 0 N control (100%) the growth rate increased to 118% up to 5 mM, then decreased to 114% at 10 mM and then to about 110% at 30 mM, which is still higher than control. Similar trend was also observed in our experiment having increase at lower concentration then decrease at higher concentration but still higher than 0 N control (Fig. 16).

It was observed that combined nitrogen in the culture media affected more adversely the nitrogenase activity of *Azolla* than its growth. The nitrogenase activity due to combined N was depressed at all the concentrations but the adverse effect was more pronounced for increase in combine-N from 0 to 14 ppm as the activity reduced to about 82% of control and thereafter it decreased only to lesser extent, and was more than 75% at 42 ppm of ammonium concentration (Fig. 16). Similarly, a sharp decline in ARA value was observed by Kumarasinghe and Eskew (1993) for initial increase in the concentration of combined N and then adverse effect was lesser with higher concentration for *A. japonica*, however more than 70% ARA value was still observed at about 2.5 mM ammonium-N (35 ppm) in the culture medium. They also observed that there was a rapid decline in the value of nitrogen derived from air (Ndfa) in *Azolla* for lower concentration (5 and 25 ppm) and thereafter its decline was lesser at 50 ppm and there was still 76% of N in *Azolla* derived from air.

The above mentioned studies and our results show that unlike other free-living or symbiotic nitrogen fixers, *Azolla-Anabaena* symbiosis shows sufficient nitrogenase activity

Estimation of N_2 Fixation by ^{15}N Dilution Technique:

The amount and rate of nitrogen fixation estimated in the laboratory may differ from the field conditions as this process is affected by the environmental conditions (Lumpkin and Plucknett 1982, Zhang et al 1987). Secondly, from the amount of nitrogen accumulated in *Azolla*, in the presence of combined N of soil or fertilizer in field conditions, it is not clear whether the nitrogen gathered by *Azolla* was taken up from combined-N source or from air through nitrogen fixation, therefore, to solve such problem ^{15}N dilution technique is used to estimate the amount of nitrogen derived from air or other sources (IAEA 1983, Eskew 1987).

Using ^{15}N dilution technique and *Lemna minor* as reference plant under field conditions, the nitrogen derived from air was 68, 75, 80, 81 and 83 for *A. pinnata* (local), *A. caroliniana*-301, *A. caroliniana*, Hybrid *Azolla* and *A. microphylla* respectively (Fig. 17a), indicating that nitrogen fixation in the presence of nitrogen in natural conditions, varies with the type of *Azolla* strain, as these strains were grown in the same environmental conditions. Since this ^{15}N dilution experiment was conducted in soil after 4 times application of ^{15}N labelled fertilizer during two years of ^{15}N labelling of *Azolla*, therefore, it was almost completely equilibrated and uniformly enriched with ^{15}N. The very well uniformly labelled soil with frequent application of labelled fertilizer and given sufficient time for attaining equilibrium with soil N pool is considered to minimize error in ^{15}N dilution technique (Kumarasinghe and Eskew 1993, Eskew 1987).

The Ndfa values under field conditions were reported to have a wide range, as the Ndfa was 75% for *A. filiculoides* and 96% for *A. nilotica* in Philippines (Watanabe et al 1981), 50-66% for *A. pinnata* and 54-61 % for *A. microphylla* in Sri Lanka (Kulasooriya et al 1987), and 79-83% for *A. caroliniana* in Hungary (Kumarasinghe and Eskew, 1993). The data indicate that there can be a wide differences in nitrogen fixation of *Azolla* depending on the *Azolla* species or strains, location, concentration of combined N in floodwater, and environmental conditions during the experiment. Evaluating the data from Austria, Belgium, Hungary and Sri Lanka, an average of about 70% or more was estimated by Kumarasinghe

strains/species was in the range of 68-83, with a average of 78%, thus this value of Ndfa is slightly higher than the average computed by Kumarasinghe and Eskew (1993), but agrees more with the value of Watanabe (1991). The Ndfa seems not to be much affected by the presence of rice plant, as in Sri Lanka it was observed, that there was no difference in Ndfa in *Azolla* whether grown as monocrop or as intercrop with rice (Kulasooriya et al 1987).

From the above discussion it is evident that the major amount of N in *Azolla* is derived from atmosphere even in the presence of combined nitrogen, thus putting a minimum drain for nitrogen on soil-N pool. So this property of *Azolla* makes its use compatible with the present day agricultural practice of using chemical N fertilizer for getting high yield of rice crops.

The rice plants growing intermixed with *Azolla* in the same sieve had higher biomass, but lower ^{15}N abundance, than the rice plants grown separately. Using rice without *Azolla* as reference plant, it was found that in rice grown intermixed with *Azolla* 21% of rice N was derived from air (Fig. 17b). The lower ^{15}N abundance and 21% Ndfa in rice grown intermixed with *Azolla* indicated that 21% of rice-N was transferred from nitrogen fixed by *Azolla*. It appears that N fixed by *Azolla* was secreted and/or released after the senescence of its older parts, and was taken up by the rice roots growing in very close contact with *Azolla* in the same sieve. The secretion of NH_3 from *Azolla* during its growth and release of its N from decaying plants has already been detailed in Review of Literature. I could not find any published work on such direct transfer of fixed N from *Azolla* to rice, however in personnel discussion, Dr. Watanabe agreed that this transfer is possible only if *Azolla* and rice roots are growing in very close contact. Similarly, a vigorous growth and increase in nitrogen content of ryegrass grown as interculture with clover due to transfer of nitrogen, most probably after mineralization of dead root tissue, was reported by Brady (1984). However, using ^{15}N dilution technique, it was confirmed that nitrogen fixed by nodulated soybean was transferred to maize and also to nonnodulating soybean when grown as intercrop with nitrogen fixing soybean (Martin et al 1991).

CARBON AND NITROGEN MINERALIZATION

Decomposition of *Azolla* in Soil:

When organic tissue, such as plant material, is added into soil, the bulk of the material undergoes enzymatic oxidation, with a release of carbon dioxide, water, different nutrients and energy Thus for release of essential nutrients and their availability to crops, the decomposition precess is essential, and CO_2 evolution is a good index of decomposition of plant material. When *Azolla* is used as biofertilizer it is usually incorporated into mud, and then allowed to decompose under flooded condition during rice growth period and after rice harvest the undecomposed *Azolla* is allowed to decompose in upland conditions in rice-wheat cropping system. To simulate both the mud and flooded conditions of rice and relatively dry or moist conditions during wheat cultivation, decomposition of unlabelled and ^{14}C labelled *Azolla* was studied at 100% saturation percentage (SP) and 30% SP of soil.

Decomposition of Unlabelled *Azolla*: The decomposition of unlabelled *Azolla* as estimated from CO_2 evolution, indicated that the rate of decomposition for both the moisture regimes (100 and 30% SP) increased at a faster rate during the first 10 days of incubation and afterwards it was slower between 10 and 40 days of incubation period (Fig. 18a). Similar trend for evolution of CO_2 was reported for incorporation of *Sesbania* into soil in the laboratory study, in which CO_2 evolution was faster up to 10th day, slightly decreased by 20th day and reached to almost a plateau stage by 40th day of incubation (Palaniappan 1992). The slow rate of decomposition at later stage of incubation may be due to depletion of readily decomposable components of plant tissue (water soluble constituents, carbohydrates, proteins etc.), during the initial period whereas lignin type complex compounds are left for decomposition which take longer time for their breakdown by the microorganisms (Alexander 1977).

Decomposition of ^{14}C-Labelled *Azolla*: Although the CO_2 evolution from *Azolla*-incorporated soil gives a good estimate of overall decomposition of organic material but differentiation of CO_2 evolved from *Azolla* or native soil organic material is impossible. As $^{14}CO_2$ evolved from ^{14}C-labelled *Azolla* gives an accurate estimation of *Azolla*-C

indicated that the mineralization of *Azolla* was faster during the first 10 days at both the moisture regimes and slowed down in the next 10 days and thereafter there was not much increase in evolution of $^{14}CO_2$ from the incubation vessels (Fig. 18b). In China, the studies of ^{14}C-labelled *Azolla* indicated that mineralization of its biomass peaked at 2-3 week after its incorporation into soil and then declined markedly by the 9th week. In the Chinese study the mineralization of *A. imbricata* was rapid as it reached to peak during 2nd week than of *A. filiculoides* having peak in the 3rd week, due to lower C:N value in the former species (Li 1984). Similarly, using ^{14}C and ^{15}N labelled *Azolla*, Wang et al (1987) found that lower C:N ratio caused a faster decomposition of *Azolla*.

In our laboratory study, the average decomposition rate for 30% and 100% SP indicated that about 45% of ^{14}C from labelled *Azolla* was lost by the end of first week, 70% by second week day and 83% by fourth week and thereafter evolution of $^{14}CO_2$ was slower (Fig. 18b). Li (1984) found mineralization of about 12% of total ^{14}C from *Azolla* by 3rd week, and 6 weeks later the mineralization was around 60% of the added *Azolla*-C. Similarly, more than half of ^{14}C-labelled *Azolla* biomass was reported to be mineralized within first 6 weeks with a peak in the 2nd and 3rd week of incubation (Wang et al 1987). However, a 5-year-study of decomposition of ^{15}N labelled *Azolla* showed a slower decomposition rate in the Chinese soil (Wen et al 1987). Singh (1992) observed faster rate of decomposition and 65-90% of *Azolla* was decomposed by third week of incubation. Lin and Wen (1987) found variable rates of decomposition in different types of soils located in different climatic zones, and concluded that decomposition rate of *Azolla* in soil is not only governed by the climate but by soil properties as well. Thus higher value, of decomposition for our experiment than Chinese results, but similar to Indian report, may be due to difference of plant material, incubation temperature and type of soil used in our studies.

In our studies faster decomposition of *Azolla* was observed at 30% SP than 100% SP, which may be due to more availability of oxygen required for oxidation of organic material and more activity of decomposing microorganisms leading to higher oxidation at moist state than at 100% water saturated conditions (Alexander 1977). In China the long-term

than 100% SP in our studies also agrees with the trend of above said work in which higher decomposition of *Azolla* was observed for upland than waterlogged condition.

Azolla-N Mineralization:

Although some of the fixed N by *Azolla* may be released into floodwater (Peters et al 1980, Rains and Talley 1979, Watanabe and Berja 1983) but most of the fixed N is released after its decomposition (Watanabe et al 1981, 1989). Therefore, understanding of mineralization of *Azolla* N is important for maximization of its use as nitrogen biofertilizer. To simulate nitrogen mineralization of *Azolla* in rice field conditions, and during wheat crop, incubation of *Azolla* was carried out at 100% and 30% SP in laboratory conditions as already explained for carbon mineralization of *Azolla*.

The laboratory studies of *Azolla* incorporation into soil and incubation at two moisture levels showed that the amount of mineral-N (NO_3-N for 30% SP and NH_4-N for 100% SP) reached to the peak value after one week of its incubation, and thereafter, it declined slightly by 10th day and then remained almost at a constant rate during the last days of incubation period (Fig. 19). The initial peak obtained by 7th day may be due to consumption of readily decomposable constituents of *Azolla* required for microbial activity, and after that lesser decomposable complex compounds may be left for slower decomposition.

During decomposition ammonia is formed from the organic material by the process of ammonification. Under flooded conditions, ammonium, which is the final and stable product of the anaerobic decomposition of organic N compounds, is derived from deamination of amino acids and aminosugars, decomposition of nucleotides and hydrolysis of urea (Watanabe 1984). The anaerobic decomposition, is largely dependent on activities of anaerobic bacteria, therefore, due to participation of limited number of microorganism, in this process, the mineralization process is slower than aerobic decomposition (Patrick 1982). This may be the major reason that lesser amount of mineral-N was formed at 100 SP and the rate of its formation also lagged behind the rate of NO_3-N in the experimental soil.

On the contrary to flooded soils, microbial decomposition of organic material in well-drained (moist) soil is accomplished by a wide group of microorganism, in which fungi play a major role (Patrick 1982). In addition to that, aerobic conditions provide oxygen for microorganism oxidizing organic matter, and also to nitrifying bacteria like *Nitrosomonas*, which convert NH_4 into NO_2 and *Nitrobacter* which convert NO_2 into NO_3 in the soil (Alexander 1977). Thus due to participation of more types and number of microbes, and higher activity of oxidizers relatively faster decomposition occurred in moist soil and due to more activity of nitrifiers higher amount of NO_3-N was formed at 30% SP than at 100% water saturation conditions, in this study. Similar to our results, the mineralization of *Sesbania* under Indian field conditions showed its peak by 10th day, and by 30th day of incubation higher amount of NO_3-N (25 ppm) was formed in soil having moisture at field capacity, whereas higher concentration of NH_4-N (15 ppm) was formed under saturation moisture regime; and about 80% of total N of the incorporated plant, was released in 10 days of incubations (Palaniappan 1992).

In a 5-year-study, the decomposition of ^{15}N labelled *Azolla* in China, showed that higher rate of its mineralization was present in upland soil than in waterlogged soil (Wen 1987). The mineralization of ^{15}N labelled *Azolla* in Madagascar, as indicated by KCl-N, showed that its decomposition was faster, during the first two weeks of incubation, than the later period in a mineral soil, whereas its mineralization was slower in the organic soil (Rabeharisoa 1994).

The relatively faster decomposition of *Azolla* in our studies resembled more to decomposition pattern observed in Indian study, as compared to Chinese and Madagascar experiments, which may be due to more resemblance between *Azolla* species, soil types and soil microflora.

Contribution of *Azolla* to Soil Humus:

The decomposition of plant material mediated by soil microorganisms results partly into CO_2 and water and partly to humus. Humus is amorphous, dark coloured, hydrophilic,

The soil fertility is controlled by complex interactions between biological, chemical and physical properties and organic matter or humus contributes to all the three factors. It increases cation exchange capacity, water holding capacity, stabilizes soil structure, provides all the essential nutrients, increases availability of trace elements by chelation to higher plants, and enhances microbial activity by providing nutrients and energy, and thus maintains life in the soil (Johnston 1991).

The analysis of soil at the end of 40 days of incubation of unlabelled *Azolla* at two moisture regimes showed higher losses of organic carbon at 30% SP moisture that at 100% SP, which may be due to higher microbial activity of decomposers in the moist soil than in water saturated soil (Patrick 1982) as explained above for *Azolla*-N mineralization. Contrary to organic carbon losses, more N losses were observed at 100% than at 30% SP which may be due to higher losses of nitrogen as ammonia volatilization because of high pH (8.0) of the experimental soil. The major factors that favour NH_4 volatilization are, high NH_3 concentration, high pH and high temperature (Patrick 1982). It has been reported that up to pH 9 the concentration of NH_3 (due to dissociation of ammonium hydroxide) increases by a factor of 10 per unit increase in pH, and these losses may be up to 60% in some flooded soils (Mikkelsen and De Datta 1979). Very high amount of ^{15}N losses from *Azolla* and algal-N were reported from flooded soil due to denitrification (Mian and Stewart, 1985). Thus incubation at warm temperature, high pH and high concentration of NH3 as mentioned for *Azolla*-N mineralization, led to higher N losses in 100 SP soil, than soil incubated at 30% SP.

Although, some of the total organic-C and N was lost, but most of organic carbon i.e. 59-63% for 30% SP and 67-76% for 100% SP, while most of the N i.e. 96-99% for 30% SP and 89-90% 100 SP was still present in the soil after 40 day incubation (Table 5).It was found that about double organic-C and 1.5 times of N, were present in soil amended with *Azolla* than control. As mentioned under Results, most of the C and N remaining in the soil were converted into humic fraction and thus increasing the fertility of the *Azolla* incorporated

organic material is essential for energy and availability of nutrients (Alexander 1977). The higher amount of N in fulvic acid as well as in humic acid in *Azolla*-incorporated soil at 30% than 100% SP may be due to more microbial activity in moist soil than water saturated soil.

The microbial activity especially of fungi decreases at high moisture level not due to water itself but due to hinderance of oxygen supply to the microorganisms (Alexander 1977), as movement of oxygen is 10,000 times slower in water than in air (Watanabe 1984). The fungi are reported to participate in formation of humus from fresh organic residues (Alexander 1977). As already mentioned for aerobic versus anaerobic decomposition, under estimation of *Azolla*-N mineralization, that during aerobic decomposition fungi are more active (Patrick 1982) thus due to higher microbial activity and more amount of fungal biomass production, higher amount of humic acids were formed in soil incubated at 30% compared without incubated at 100% SP.

USE OF *AZOLLA* IN RICE

Although *Azolla* can be used as biofertilizer for other crops, but it has been used as green manure mainly for rice crop in different countries (Lumpkin and Plucknett 1982, Liu 1979, Lumpkin 1987a,b, Watanabe 1994). To investigate its effect on rice yield, floodwater properties and fertilizer-N use efficiency, different greenhouse and field experiments were performed.

Greenhouse Studies:

Effect of *Azolla* and Blue-Green Algae (Pot Expt. 1): In the Results it was mentioned that *Azolla* grew without any pest problem. Since *Azolla* was grown in pots placed at a distance of about two feet apart, therefore, the temperature and humidity did not rise too much due to good aeration. The combination of both high temperature and humidity are considered conducive for pest and fungal attack (Lumpkin and Plucknett 1982, Van Hove 1989, Roger and Watanabe 1986). Secondly half of the *Azolla* was incorporated whenever it covered the water surface so it did not reach the stage of overcrowding, as overcrowding

The growth rate of *Azolla* remained high upto second week of November and then slowed down during last two weeks of November and in December. The better growth of *Azolla* during early period of rice growth than in the later part may be due to lesser shading by younger rice plants thereby lesser hinderance of light but making the temperature more desirable and also due to longer photoperiod during August to October. During November and December the rice plants have a bigger canopy so due to their shade, lower temperature than optimum and shorter period the *Azolla* growth was reduced. The good effect of longer photoperiod was reported by Peters and Ito (1984), and preference of *Azolla* for partial shading during severe summer, while over-shading by the rice canopy during later stages of rice growth was reported by Singh (1992).

The higher value for flag leaf area and second leaf area for urea than other treatments seems due to enhancement of vegetative growth by its N availability to rice, and leaf area index is reported to be positively correlated with rice yield (De Datta 1981). The slightly higher tiller height for urea, and more number of fertile tillers for urea and *Azolla* inoculated treatments, seems due to more availability of N to rice than from dead *Azolla* or BGA, and a positive effect of N on tiller height and fertile tillers has also been reported (Lumpkin and Plucknett 1980, Singh 1979, 1989, Singh and Singh 1987, Yanni et al 1994). The higher number of total tillers per pot for urea may be due to availability of its N at early stage of rice growth leading to higher number of tillers (De Datta 1981) and increase in tiller number at higher dose of N and *Azolla* was reported by Yanni et al (1994). The higher number of sterile tillers for BGA and control may be due to insufficient supply of N to rice plant as number of filled spikelets per panicle is correlated with the amount of N absorbed at panicle development stage (De Datta 1981), and higher sterility in control than for urea and *Azolla* was also observed by Singh and Singh (1987). The longer panicles for urea and *Azolla* incorporated treatments may be due to higher availability of N for these treatments as compared to other treatments. The increase in rice panicle weight for urea and *Azolla* was reported by Mahapatra et al (1987) and Singh and Singh (1987).

The higher rice yield for urea treatments than *Azolla* (Table 7b) may be due to faster

release of *Azolla* N was comparatively slower. According to Singh (1992), the availability of *Azolla* N to first rice crop is about 70% of ammonium sulphate.

We used mixture of nitrogen fixing blue-green algae for inoculation, as the use of algal mixture is reported to offset the ecological and edaphic dangers to a particular strain in a given locality and is helpful for establishment of any one of the strain suitable to that situation (Roger and Kulasooriya 1980).

Although blue-green algae has been reported to increase rice yield at most of the experimental sites (Roger and Kulasooriya 1980) but in our experiment it did not cause any increase in rice yield rather there was some negative effect. The poor result for BGA may be due to lesser growth after its inoculation, as the inoculated types disappeared after a few days. The disappearance may be due to their competition with the native algae of the experimental soil or consumption by grazers, as no insecticide was applied alongwith BGA. The eating up of inoculated BGA has been reported by Roger and Kulasooriya (1980). In addition to that the algal growth is reported to increase the floodwater pH during noon time and leading to N losses through ammonia volatilization (Mikkelsen and De Datta 1979). Thus the N losses from rice ecosystem by BGA, led to negative effect on rice yield. It was also reported that inoculation of BGA in India did not increase rice plant height, but increased rice yield to some extent and the response was poor than *Azolla* (Singh and Singh, 1990).

Since BGA alone caused a slight decrease in rice yield, therefore the higher yield for 40 kg N/ha and 60 kg N/ha alongwith BGA seems due to positive effect of urea.

The dead *Azolla*, equal to live inoculum, also did not increase rice yield which may be due to very low amount of N in its dead tissue.

As compared to control the rice straw yield was 33-66% higher for urea while 10-21% for *Azolla* (Table 7b) because urea N was more available at early stages of rice growth and *Azolla* needs some time for release of its N (Singh 1990, 1992). Since higher values for leaf area, tiller height and total tillers was observed for urea treatments, therefore, a higher vegetative growth was responsible for higher rice straw yield for the urea treatments than

than organic N source on rice straw formation. Mahapatra et al (1987) has also reported a higher yield of straw for urea than *Azolla* when both were applied at the rate of 25 kg N/ha.

Unlike rice straw yield, the difference in grain yield for urea and *Azolla* treatment was lesser. As compared to control the grain yield was 36-39% higher for *Azolla* while 51-82% for urea. The better performance of *Azolla* for increasing grain yield than for straw yield may be due to availability of more period for *Azolla* decomposition to release its N during grain formation (Singh 1992). According to De Datta (1981), nitrogen absorbed by the rice plant during panicle development increases filled spikelet per panicle and absorbed after flowering increases grain weight.

The flag leaf area and second leaf area was higher for *Azolla* than control, and leaf area index is reported to be positively correlated with grain yield due to increase in photosynthesis as 75-80% of carbohydrates in grain are photosynthesized after flowering (De Datta 1981). Thus due to more leaf area index, lower sterility in tillers and more nitrogen supply than control and BGA, a higher grain yield was obtained for *Azolla* intercropped and incorporated treatments.

The 10% higher yield of rice straw for *Azolla*+FYM than only *Azolla* may be due to availability of nitrogen from FYM, as it contains about 0.5-1.0% N (Gaur 1992). However, the grain yield for both these treatments was similar which may be due to application of small amount of FYM (2t/ha) containing about 14 kg N/ha and major portion of which was utilized for straw formation and probably not much nitrogen was left for grain formation. Similarly the grain yield was almost equal for *Azolla* medium and only *Azolla*, indicating that like low amount of FYM it also did not help in *Azolla* growth and thereby did not increase the rice yield. A very low amount of FYM was applied during this experiment to reduce the expenditure, but it did not gave the desired effect, and quite larger amounts of FYM i.e. 5-10 t/ha are recommended for *Azolla* cultivation (Roger and Watanabe 1986).

Effect of *Azolla* and Urea (Pot Expt. 2): The rice straw yield was higher for *Azolla*+FYM and urea applied at 50 kg N/ha. As already explained for pot experiment 1, that nitrogen availability at initial stages of rice growth tends to improve vegetative growth

statistically similar yield to control as this was not sufficient amount to exert any significant effect on rice yield.

Unlike previous experiment No.1, higher grain yield was obtained for *Azolla*+FYM and *Azolla* intercropped and incorporated treatment than urea. The higher grain yield for *Azolla*+FYM in this experiment may be due to higher amount of FYM (12 t/ha) used in this experiment. The higher grain yield for FYM+*Azolla* incorporated than FYM+*Azolla* cover may be due to faster decomposition of *Azolla* in soil after its incorporation thereby releasing more N for rice plant. The decomposition of *Azolla* incorporated into soil was reported to be 50% higher than left as cover (Singh 1992). Similarly the N recovery in rice of ^{15}N labelled *Azolla* incorporated into soil was 5 times higher than leaving it as cover in pots (Watanabe et al 1981).

Unlike previous experiment No. 1, the grain yield (although statistically similar) was higher for *Azolla* than 50 kg N/ha. Secondly, the overall rice biomass (straw+grain) produced per pot was nearly double in this experiment than the previous experiment. The almost double yield of rice for this experiment is that, the rice was transplanted about 3 weeks ahead of the last year experiment. Thus more growth period and days of longer photoperiod were available for rice growth.

The higher rice yield in longer photoperiod and due to longer growth period for tall varieties has been reported by De Datta (1981). The second advantage of longer growth period was for *Azolla*, as more growth was possible and more time was available for its decomposition and release of N for rice. Thus better response of *Azolla* than the previous year's experiment was observed during this year.

Effect of *Azolla* and Farmyard Manure (Pot Expt. 3): In the previous experiment No. 2, higher rice grain yield was obtained for *Azolla*+FYM than other treatments and it was not clear whether the major role is of *Azolla* or FYM. In this experiment a separate treatment of FYM was included. As compared to control the rice grain yield increase was 72% and 73% for FYM (6 t/ha) and urea (60 kg N/ha) indicating equal benefit for both the doses used for each of these sources. FYM is reported to have 0.5-1.0% N (Gaur 1992) thus

The FYM and urea gave equal increase in grain yield, but FYM+*Azolla* gave higher than either of these applied individually, indicates that FYM enhanced *Azolla* growth and nitrogen fixation. The addition of superphosphate along with *Azolla* increased grain yield but was not statistically significant, which may be due to its lower effect on *Azolla* growth as split application of P is considered more useful than single application (Watanabe 1982, 1994).

Effect of *Azolla* on Fertilizer-N Use Efficiency (Pot Expt. 4): The almost absence of algal growth in floodwater covered by *Azolla* may be due to competition for nutrients, less alkaline pH and hinderance of light. Since mostly blue-green algae were growing in non-*Azolla* pots and they prefer alkaline pH (Roger and Kulasooriya 1980) therefore optimum pH was not available for BGA growth. The reduction in algal growth by *Azolla* cover has also been reported by Tuan and Thuyet (1979). About 10% of total height was reported to be reaching below *Azolla* mat (Krock et al 1988), thus insufficient availability of light below *Azolla* mat may be the main reason for almost no algal growth in *Azolla* cover pots. As only a small fraction of light passed through *Azolla*, heating effect of solar radiation was also reduced, which led to lower temperature of floodwater, and temperature difference of 3°C for *Azolla* mat was observed in China (ZAAS 1975).

The lower soil-water interface and floodwater pH of *Azolla* covered water may be due to respiration of *Azolla* roots and excretion of hydrogen ions for ion exchange (Brady 1984). Secondly the very low light available below *Azolla* mat led to reduction in algal growth. The algae is reported to consume CO_2 and H_2CO_3 of water causing a higher pH at noon time (Lumpkin and Plucknett 1982, Mikkelsen and De Datta 1979, Krock et al 1988).

The taller tillers, more number of tiller per pot and higher straw yield for urea may be due availability of its N at early stage as detailed for pot Expt. 1. The higher rice grain yield for *Azolla* incorporated than cover was due to its faster decomposition after incorporation into soil than left as floated, as discussed under previous pot experiment 2.

The *Azolla* cover led to 29% higher grain yield than control, which may be due to release of ammonium into floodwater (Talley and Rains 1980) or after release of its N from

reported by Watanabe (1987) in INSFFER trials of different countries and recently by Kulassoriya et al (1994) and Yanni et al (1994).

The higher grain yield for *Azolla*+urea treatments than only urea treatments for same dose of urea application, is of practical significance. As compared to control the increase in grain yield was 48% for 30 kg N/ha, increased to 67% alongwith *Azolla* cover and increased further to 111% alongwith *Azolla* incorporated, treatment. Similarly for 60 kg N/ha it increased from 115% to 156% for urea+*Azolla* incorporated treatment. However, benefit of *Azolla* was more pronounced for lower dose of urea application, indicating that use of *Azolla* alongwith a low dose of fertilizer N (30 kg N/ha) can lead to increase in rice grain yield equivalent to 60 kg N/ha. The good effect of *Azolla* alongwith chemical-N fertilizer at low doses was also reported by other workers (Kumarasinghe and Eskew 1993, Yanni et al 1994, Watanabe 1987).

The total N uptake in rice plant and retention in soil was higher for *Azolla* and *Azolla*+urea treatments than urea only, indicating more positive balance of nitrogen in soil-plant system due to *Azolla*. Similarly the ^{15}N recovery of applied urea was higher for urea+*Azolla* treatment than urea only, indicating improvement in fertilizer-N use efficiency due to *Azolla* application. Similarly a higher ^{15}N recovery of urea in rice plant due to *Azolla* was observed by other workers (Kulasooriya et al 1994, Kumarasinghe and Eskew 1993). As more fertilizer-N was taken up by rice plant and more of urea-N was retained in soil, so fertilizer-N losses from plant+soil were reduced by *Azolla* application, being 41-42% for urea while 19-31% for urea+*Azolla* treatment. The lower losses for urea+*Azolla* than only urea may be due to lowering of floodwater pH by *Azolla*, and higher floodwater pH is reported to increase N losses by ammonia volatalization (Mikkelsen and De Datta, 1979, Roger et al 1993).

Field Studies:

The field studies of *Azolla* use in rice crop indicated similar trend as of greenhouse study but sometimes, the difference between various treatments were less prominent because

while for the same treatments higher grain yield was obtained for urea (50 kg N/ha) than *Azolla*+FYM in the field experiment 2 (Table 12). The higher rice yield for *Azolla*+FYM than for 60 kg N/ha during first year may be due to higher amount of FYM (12 t/h) used alongwith *Azolla*. It was also observed that about 12 t fresh *Azolla*/ha was produced for FYM application as compared to 7.5 t/ha without FYM application. Thus higher amount of *Azolla* led to increase in rice yield for *Azolla*+FYM treatment. The lower response of urea in this experiment may be due to higher level of available N in this soil, which was lying fallow before the experiment. A poor response of fertilizer N in nitrogen rich soil especially on tall varieties like Basmati is reported by De Datta (1981). During the second year the soil was exhausted after rice and wheat crop, therefore, more response of even lower dose of FYM and urea was observed.

Evaluation of *Azolla*-N Uptake and Yield Response (Field Expt. 3 and 4): Both these experiments had same treatments except that in case of Expt. 3, *Azolla* and urea were incorporated at 40 and 80 DAT, while at 10 and 40 DAT for Expt. 4. In Expt. 4 higher response of *Azolla* was observed on rice as compared to Expt. 3, because more time was available for decomposition of *Azolla* to release it N for rice crop, as earlier incorporation of *Azolla* than its late incorporation was reported to be more useful for increasing number of tillers and rice yield (Singh and Singh 1987). They also observed that incorporation of *Azolla* after 4 weeks of rice transplanting did not increase total dry biomass and grain yield of rice. Similar to *Azolla* response, rice grain yield was low for late incorporation (40 and 80 DAT) than earlier incorporation (10 and 40 DAT) of urea. The later application of nitrogen fertilizer did not improve tillering of rice and a very small amount of it is taken up by rice as most of the rice N is taken up between second and third month after its transplanting (De Datta 1981). Thus N applied after 60 days of its transplanting was of little value for rice.

The ^{15}N recovery in rice for *Azolla* in Expt. 3 was lower than in Expt. 4, because of late incorporation (40 and 80 DAT) in the former case. In addition to lesser time period available for *Azolla* decomposition due to its late incorporation, the lower soil temperature

Effect of P Enriched *Azolla* on N Availability (Field Expt. 5): The floodwater P concentration of *Azolla* nurseries remained higher as long as its application was continued. (Fig. 23), which may be due to regular and more frequent addition of superphosphate at a short internal of 2-4 days, indicating that floodwater P can be maintained at a much higher level with frequent applications. Similarly, the regular and split application of P on alternate days was found to maintain a higher floodwater P as compared to control or its application at one time only (Watanabe et al (1980). The lowering of floodwater P to original level after stoppage of its application within a week may be due to attainment of equilibrium conditions due to absorption of P from liquid to onto soil particles (Watanabe et al 1980) or reaction with calcium and iron or other ions (Brady 1984). A similar decline in floodwater P to equilibrium level within a week, was also observed in Philippine soils (Ali and Watanabe 1986).

Although the floodwater P remained high in 2, 4, and 6 P nurseries during *Azolla* growth (Fig. 23), but *Azolla* yield and its nitrogen contents did not increase significantly. The average *Azolla* growth inspite of regular P addition indicated that in addition to P some other nutrient was limiting the *Azolla* growth in this soil, and requirement of all the essential nutrients at the optimum concentration has already been discussed in '*Azolla* Survey'.

The rice straw and grain yield were statistically similar for 15, 30 and 45 kg N/ha but increased with an increase from 15 to 30 kg but not for 30 to 45 kg N/ha. The lower response at lower doses and no response at higher dose indicated that this soil had sufficient available N. Similarly the application of *Azolla* grown at different P levels did not show the expected response. The low response of fertilizer-N on fertile soil is also reported by De Datta (1981) and *Azolla* response was reported to be better in low fertility soils (Loudhapasitiporn and Kanareugsa, 1987).

Like rice grain yield the ^{15}N recovery in rice was also less affected for increase in urea doses particularly at the higher level, however ^{15}N recovery was better for 2P and 4P *Azolla* than 0P indicating a useful effect of *Azolla* grown at higher P level. It is reported that increase in floodwater P usually results in higher P in *Azolla* (Ali and Watanabe 1986,

Effect of *Azolla* on Urea-^{15}N Recovery (Field Expt. 6): The floodwater pH was higher for non-*Azolla* than *Azolla* applied plots (Fig. 24), which may be due to reduction in algal growth by *Azolla* shading as already explained for rice pot experiment 4. The same pattern of decrease in pH for *Azolla* cover in the field study indicated that pot studies are also useful.

The higher straw yield for urea incorporation at transplanting than its application 2 weeks after transplanting, was due to availability of its N at the early stage of rice growth, as already detailed for rice pot experiment 1. On the contrary to rice straw yield, grain yield was higher for urea application at 2 weeks after rice transplanting that at 0 DAT. The higher grain yield for urea application at 14 DAT alone and along with *Azolla* indicated that a later application was more useful for grain yield. As already discussed, that nitrogen available at later stages of rice growth leads to higher grain yield in rice (De Datta 1981). Although the rice root biomass was statistically non-significant but it was slightly higher for *Azolla*+urea treatment which may be due to more supply of N from both the sources. An increase in rice root biomass with an increase in nitrogen was also observed by Mian and Stewart (1985). The total N uptake in rice straw, grain and root was generally higher in case where biomass of these plant parts was also high, indicating a positive correlation between biomass and total N yield. The N yield was higher in straw for urea application at transplanting, while in grain it was higher for urea application at 14 DAT alone or with *Azolla*, which may be due to differential availability of N to rice plant at both the rice growth stages as explained for rice straw and grain yield. An increase in N uptake by rice with an increase in fertilizer-N was reported by De Datta (1981), while for *Azolla*-N by Mian and Stewart (1985).

The ^{15}N recovery of labelled urea was higher for straw, while of *Azolla* in grain, which may be due to availability of urea-N at early stage and *Azolla*-N at later stage of rice growth. It has been reported that *Azolla* N is released slower than chemical-N (Singh 1990, Singh and Singh, 1987). ^{15}N recovery in rice grain was higher for urea-N applied alone or with *Azolla* at 14 DAT than applied at transplanting, indicating that more of N was take up if applied at later stage than at rice transplanting stage. The higher ^{15}N recovery in rice grain

The greater amounts of ^{15}N from *Azolla* and urea were retained in top soil (0-15 cm) than in bottom layer (15-30 cm). The vertical distribution of mineral N in a Philippine soil after 2-3 months of fertilizer application also showed that most of the N was localized in the top (0-20 cm) layer as compared to lower layers (Watanabe et al 1977). The higher amount of ^{15}N from *Azolla* in soil may be due to its residual N left over because of slower availability than urea to rice plant. Similarly a significantly higher ^{15}N (37-50%) was observed in soil for *Azolla* than urea (22%) in a field study by Watanabe et al (1989).

The total ^{15}N recovery in plant+soil was higher for urea incorporated at transplanting and urea broadcasted at 14 DAT + *Azolla* cover. The higher ^{15}N recovery in plant+soil or lower losses of N from the system for these two treatments indicated that incorporation of urea reduced N losses, while the presence of *Azolla* cover also reduced N loses from the rice ecosystem. The less losses of applied N due to incorporation has been reported by De Datta (1981), while the reduction in N losses for *Azolla* cover was also observed in similar studies in other countries (Kumarasinghe and Eskew 1993).

RESIDUAL EFFECT OF *AZOLLA* ON WHEAT

The residual effect of *Azolla* and *Azolla*+FYM was higher than urea for increasing the wheat grain yield in pot Expt. 1R, and field Expt. 1R. Similarly much higher grain yield was obtained for *Azolla* alone and for *Azolla*+urea treatments in pot experiment 2. These results show that organic source of nitrogen was superior for residual effect than chemical-N fertilizer, which may be due to improvement of soil fertility by the organic source as it provided a balanced nutrients than inorganic source. Secondly, the nitrogen and other nutrients from organic source are released slowly and regularly, therefore, losses of N from these sources are less. The improvement in soil fertility by organic manures leading to more residual effect has been reported by Palaniappan (1992).

Although there were same treatments for field experiment 2R and 3R, but residual effect of *Azolla* as well as urea were significantly higher for 2R than 3R, because *Azolla* and urea were applied at later stages of rice growth (40 and 80 DAT) and most of the N was still

slower decomposition of *Azolla* and its nitrogen was conserved and then released during wheat growth period when temperature was favourable for its decomposition (Fig. 22).

The higher residual effect of *Azolla* than urea seems due to more conservation of its nitrogen than urea-N, as in flooded conditions fertilizer-N is reported to be lost more as ammonia than organic sources (De Datta 1981). A higher residual effect of *Azolla* than chemical nitrogen fertilizers, supplying almost equal amounts of N, was observed on subsequent wheat crop by Kolhe and Mitra (1987). Similarly a significantly higher residual effect on the 10th crop of rice was observed for *Azolla* and *Sesbania* than urea, when they were applied to previous rice crops (Watanabe and Ventura 1992).

The ^{15}N recovery in wheat for *Azolla* and urea applied 40 and 80 DAT to rice in Expt. 2R, indicated that it was higher in straw from urea while higher amount was recovered from *Azolla* into wheat grain, indicating more availability of *Azolla*-N at later stage of wheat growth due to slower decomposition at cooler temperature in the early growth period of wheat. It may be noted that although ^{15}N recovery in wheat was more for urea than *Azolla* but grain yield was higher for *Azolla* than urea, which may be due to a balanced release of nutrients from *Azolla* than urea, and the balanced fertilizers (NP) are reported to give about 50% more yield than application of nitrogen fertilizer only, in our local conditions (Saleem 1994). Secondly the residual effect on yield was found to be correlated with total N uptake by the plant (Watanabe and Ventura 1989), and similarly the grain yield was more correlated with total N yield than ^{15}N recovery in grain as observed in Expt. 3R.

The residual effect for urea and *Azolla* grown at different P levels (Expt. 4R) indicated statistically similar residual effect for wheat yield, which may be due to low amount of *Azolla* incorporated during rice crop, and insufficient *Azolla*-N was left over to cause any significant effect on wheat yield.

The residual effect of *Azolla* and urea in field Expt. 5R, showed that higher wheat grain yield was obtained for urea applied at 14 DAT alone, *Azolla* alone, and urea 14 DAT+*Azolla*, than urea applied at rice transplanting. It indicates that urea applied 14 DAT was more left for wheat; and *Azolla* alone and also with urea resulted into better residual

The total N yield in wheat grain for field Expt. 5R, showed that it was higher for the same treatments which led to higher grain yield, indicating a positive correlation between residual effect on grain yield and total N uptake. A similar correlation was also observed by Watanabe and Ventura in their studies (1989).

The ^{15}N recovery of *Azolla* and urea applied to rice was only 2-5% in wheat plant (straw+grain), and it was slightly higher for urea applied at 14 DAT. Whereas higher ^{15}N was retained in soil from *Azolla*, which may due to slower decomposition of its lignified tissue or immobilization of its N into stable organic matter as already explained for "*Azolla* contribution to soil humus". In the similar studies in other countries it was also found that ^{15}N recovery of *Azolla* and urea was also low and ranged 0.8-4.4% in the second crop because only a part of the immobilized N was mineralized during second crop (Kumarasinghe and Eskew 1993).

After the 2nd crop i.e. wheat crop, 27-60% ^{15}N was present in soil (top+bottom) for various treatments in our studies. Similarly ^{15}N remaining in soil at the end of the two crops varied from 11-64% in different countries having similar studies (Kumarasinghe and Eskew 1993). The maximum amount of ^{15}N (60%) in both the top and bottom layer of soil for *Azolla*, followed by *Azolla*+urea (average 40%) and minimum for only urea (average 29%) indicates that *Azolla* helps in build up of soil N pool. Similarly more contribution of *Azolla* for building of N in the soil than urea, resulting into better soil fertility, was also observed in other countries (Kumarasinghe and Eskew 1993).

POSSIBLE FERTILIZER SAVING

In Pakistan rice is grown in more than 2 million hectare (IRRI, 1994). Normally the farmers use 1 bag (50 kg) of urea (46% N), at transplanting and then another bag at panicle initiation stage per acre, thus total 46 kg N/acre corresponding to about 114 kg N/ha is used for rice. Taking 2 million hectare as the rice area, the nitrogen consumption comes to about 238367 tons/rice crop in Pakistan. On the average, *Azolla* caused rice grain yield equivalent to application of 30 kg N/ha, corresponding to a saving of 1/4 of the present consumption

Rs. 230/bag of urea (Rs. 10/kg N) Rs. 238 million (US$ 7.5 million) can be saved through *Azolla* use per rice crop in Pakistan.

In rice-wheat cropping system, in more than half of this area, wheat is sown after rice and 2 bags of urea are also applied for each acre of wheat crop. If we assume a 10% saving of N due to residual effect of *Azolla*, then about 11919 tons of nitrogen fertilizer costing about Rs. 119 million (US$ 3.75 million) may be saved per wheat crop.

Thus in Pakistan a big amount of nitrogen fertilizer (35606 tons) costing a huge amount of capital (Rs. 357 million = US$ 11.25 million) can be saved each year through the use of *Azolla* as biofertilizer in rice-wheat cropping system.

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