Role of *Azolla* in Different Ecosystems

A Thesis

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By

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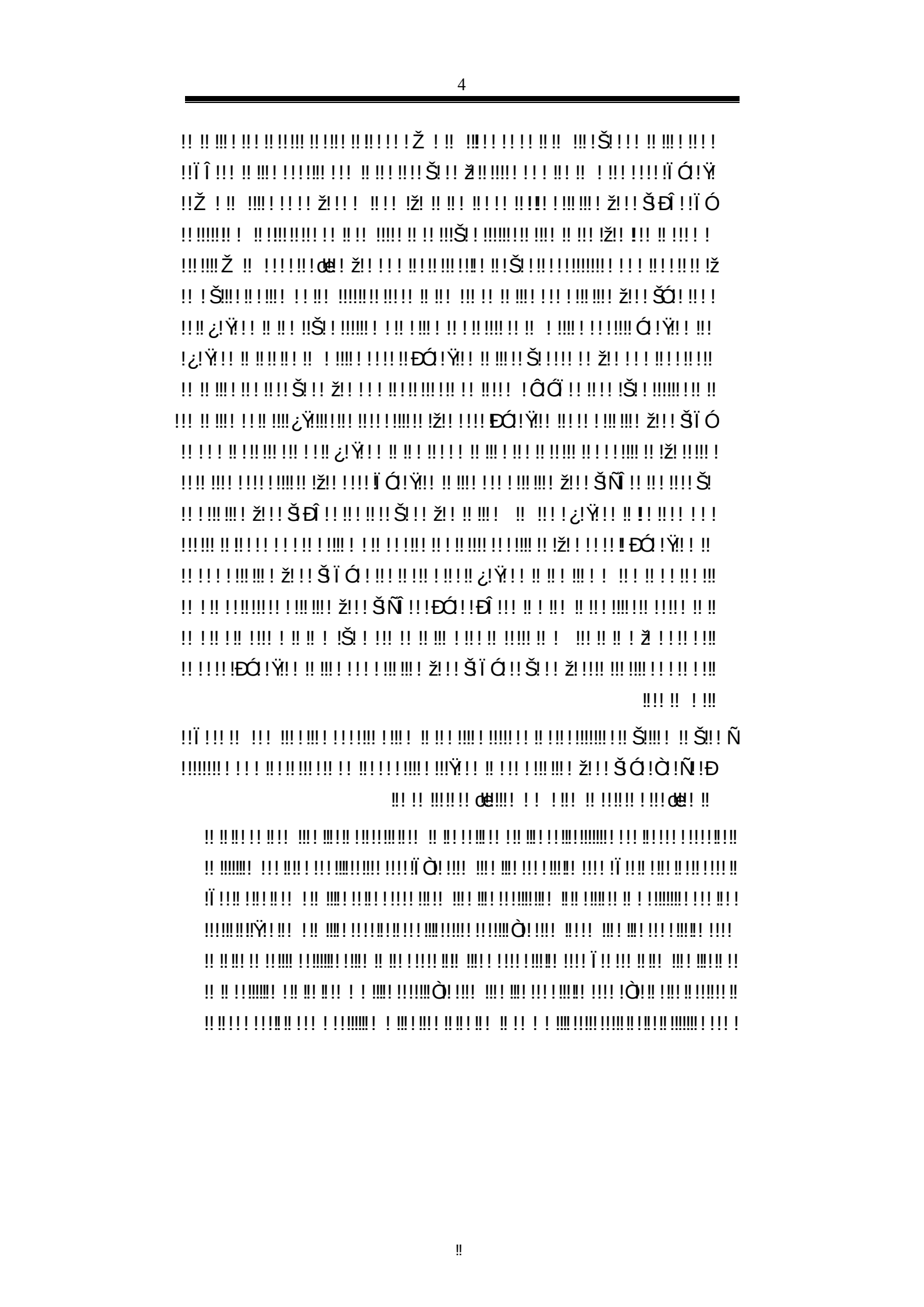

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INTRODUCTION

Azolla is a small free floating aquatic fern its native to Asia, Africa, and the Americas. It lives in swamps, ditches, and even in lakes and rivers where the water is not turbulent (**Lumpkin and Plucknett, 1982**).

Azolla leaf consists of two lobes, an aerial dorsal lobe, which is chlorophyllous, and a partially submerged ventral lobe. Each dorsal lobe contains a leaf cavity, which houses the symbiotic *Anabaena azollae*. However the dominant symbiont in association with *Azolla* sp. is more closely related to *Nostoc* spp. than to free living *Anabaena* spp. (**Peters 1991 and Wagner 1997**).

Nina, (1999) reported that, fast growing floating and submerged freshwater macrophytes are used commercially all over the world in aquaculture systems to produce protein rich feed for animals, green manure; remove nutrient in waste water treatment, and biogas production.

Azolla have a symbiotic association with the N_2 fixing cyanobacteria *Anabaena azollae*. It can fix 30-60 kg N ha^{-1} in 30 days. Also, the fern is used as an important biological source to improve the nitrogen balance of rice fields. Where, it contains 3-6% N dry weight and it could double it's biomass every 3-5 days (**Watanabe, 1982**).

The aquatic nature for *Azolla*, its rapid growth, high nitrogen content, and its ability to increase rice yield. *Azolla* has been used as a green manure in lowland rice cultivation for centuries especially in countries like China and Vietnam (**Kulasooriya *et al*., 1984**).

-REVIEW OF LITERATURE

2.1. The Fern:

The *Azolla* macrophyte called frond ranges from 1.0-2.5 cm length in species *Azolla pinnata* and to 15 cm or more largest, in *Azolla nitolica*. The *Azolla* saprophyte consists of a horizontal to vertical main rhizome bearing individual roots or root bundles at the branch points and alternately arranged bilobed leaves with an endophytic cyanobacterium. Achlorophyllous roots are initiated at branch points along the rhizome by single mother cells which undergo periclinical division, and bear root hairs up to 1 cm long which emerge from under a root cap. Root hairs arise from the tangential division of epidermal cells on the lower half of the root. Unbranched, adventitious roots hang down into the water from nodes on the ventral surfaces of the rhizomes, the roots absorb nutrients directly from the water, through in very shallow water they may touch the soil, deriving nutrients from it (**Peters, 1977; Warmbrodt and Evert, 1978 and Lumpkin and Plucknett 1980**).

The leaf consists of two lobes, a thick aerial dorsal lobe and a thin ventral lobe occasionally of a slightly larger size. The dorsal lobe is chlorophyllous, except in the transparent margin, and contains the *Anabaena* colony within a basal cavity 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 cell stomata. The interior surface of each leaf cavity is lined with envelope and covered by a mucilaginous layer of unknown composition which is embedded with filaments of *Anabaena Azolla*e and permeated by multicellure transfer hairs (**Konar and Kapoor, 1972; Peters, 1976; Becking, 1978; Robins *et al*., 1986; Lumpkin, 1987 a and Shi and Hall, 1988**).

2.2. *Azolla- Anabaena* Relationship:

The *Anabaena* – *Azolla* system is a symbiotic association between pteridophyta and blue green algae *Anabeana azollae*. The host

Azolla contains chlorophyll a and b and carotenoides, the symbiont *Anabaena azollae* contains chlorophyll a, phycocyanin, allophycocyanin and phycoerythrocyanin. The *Azolla-Anabaena* association exhibits Calvin cycle (**Tyagi *et al*., 1980**).

In *Azolla* – *Anabaena* symbiosis, N_2 is fixed by the symbiont and then transported to the host. The host *Azolla* incorporates this newly fixed N_2 into amino acids. Probably these amino acids, along with reductant and photosynthate, are then supplied to the symbiont. A study with ^{15}N (**Peters *et al*., 1980 a**) revealed that the isolated symbiont exudes up to 50% of its fixed N_2 into incubation medium as ammonia, this observation led to several studies on the enzymes in the *Anabaena* and *Azolla* that are involved in ammonia assimilation. It was found that both organisms are capable of assimilating ammonia via the enzymes glutamate synthetase,glutamate synthase and glutamate dehydro-genase. On the other hand, the enzyme nitrogenase which is believed to occur in heterocysts of the *Anabaena* symbiont is capable of reducing N_2 and other substrates such as acetylene (**Ray *et al*., 1978 and Ladha and Watanabe, 1984**).

Anabaena *Azolla*e has three kinds of cells: vegetative cell, heterocyst and akinetes. The heterocyst is the actual site of N_2–fixation. A remarkable feature of symbiotic *Anabaena azollae* is the very high heterocyst frequency, the distance between 2 heterocysts is about 3-5 vegetative cells. In free living blue green algae, that distances is 15-30 vegetative cells. The blue green algae *A. azollae* consists of unbranched trichomes. In very young leaves, trichomes lack heterocysts. As the leaf matures *Anabaena* increases its number and heterocyst frequency and become able to fix atmospheric nitrogen symbiotically and supplies the fixed nitrogen to the fern. Heterocysts gradually increase in frequency untill they comprise 30-40% of the algal cells, Mature trichomes on average consist of 60.9% vegetative cells, 23.1% heterocysts, and 16% akinetes (**Hill, 1977; Ladha and Watanabe, 1982; Van Hove, 1989 and Maejima *et al*., 2002).**

2.3. Growth Rate of *Azolla*:

Peters *et al*. (1980b) were able to obtain relative growth rates (RGR) under optimal laboratory conditions of 0.355 to 0.390 g/g day. Where **Talley and Rains (1980b)** achieved maximum RGR using *Azolla filiculoides* in phytotron experiments of 0.245-0.277 g/g day in temperature regimes of 25/15 °C to 35/25 °C. They found that the nutrient solution was changed every 7 to 14 days depending upon RGR.

Doubling time as rapid as three days or less will be difficult to achieve or maintain in field cultivation, the doubling time of *Azolla* in controlled environment of an "*Azolla* factory" or under laboratory conditions may be reduced to as little as 30 hours (**Ito and Watanabe 1983**).

Sucrose is a primary end product in *Azolla*, a regime of 2% concentrations glucose, fructose, sucrose or maltose in the nutrient solution increased the growth rate of *Azolla*. Where certain sugars (such as sucrose) could increase the productivity of *Azolla* by 183% over the sugar-free control. (**Nickell, 1961; Kim and Kim, 1967 and Lumpkin and Plucknett, 1982**).

Hechler and Dawson (1995) found that high plant density decreased specific nitrogenase activity per unit biomass and per unit area. They found that the optimal plant density for nitrogenase activity in *Azolla* caroliniana was 50-100g dry weight/m^2.

El-Araby *et al*. (1999) found that *Azolla pinnata* is superior than *Azolla filiculoides* when it gave higher growth N,P and K contents in all tested growth periods. Nitrogenase of *Azolla pinnata* was also more active than that of *Azolla filiculoides*. They recommended *Azolla pinnata* for applications as a green manure.

2.4. *Azolla* Taxonomy and Distribution:

The name of *Azolla* is derived from the two Greek words, Azo (to dry) and Ollyo (to kill) thus reflecting that the fern is killed by drought.

The genus *Azolla* Lamarck (established by **Lamarck, 1783**) belonged to the family salviniaceae and consisted of two subgenera and six living species. a- subgenus *Euazolla* included four species *Azolla filiculoides*, *Azolla caroliniana*, *Azolla microphylla* and *Azolla mexicana*. b- the subgenus Rizosperma included two species: *Azolla* pinnata with simple gloshidia and *Azolla nilotica* with no glochidia.**(Lumpkin and Plucknett, 1980).**

The subgenus Rizosperma is characterized by the presence of papillae all over the vegetative body, the presence of nine megaspore floats, and the presence of simple glochidia; whereas subgenus *Euazolla* is characterized by the possession of papillae only on the leaves and some branches, the presence of three megaspore floats, and the presence of well developed, septate glochidia with terminal anchor-like structures (**Lumpkin and Pluckentt, 1982**).

Tan *et al*. (1986) suggested that it is more appropriate to place *Azolla* in the monotypic family *Azolla*ceae. They propose that both subgenera and sections should be used in the supraspecific taxonomy of *Azolla*, with *Azolla* being divided into sections:-

I. Section *Azolla* should include (*A. filiculoides*, *A. mexicana*, *A. caroliniana* and *A. microphylla*).

II. Section Rizosperma should contain only *A. pinnata*. Moreover, they propose that *A. nilotica* can be placed in a new subgenus,

tetrasporocarpia. They Justify this separation of *A. nilotica* into its own subgenus on the bases that it has the unique habit of producing sporocarps in fours, that it has a chromosome number of 2n=52 (Whereas *A. pinnate* has 2n=44 like the species of sect. *Azolla*), and that it is evolutionarily more distant from the species of section *Azolla* than is *A. pinnata*.

They added another species, *Azolla rubra* to section *Azolla*, they preferred to regard it as a variety of *A. filiculoides*.

Some recent developments in the taxonomy of *Azolla*. **Saunders and Fowler (1992)** revised the taxonomy of section Rizosperma based on multivariate statistical analysis of ultrastructural, gross morphological, and anatomical characteristics. They more clearly delimited the characteristics of *A. pinnata* and *A. nilotica*. They named and described three distinct subspecies of *A. pinnata*, namely, subspecies pinnata, *asiatica* and *africana*.

Table (1): Taxonomy of *Azolla* according to Saunders and Fowler (1992).

Section	Species	Origin	Megasporocarps	Macrosporocarps
Rhizosperma	A. nilotica	Central-East Africa	9-Floats	Small glochidia of Massulae
Rhizosperma	*A. pinnata*	Asia, Oceania, Africa	9-Floats	No hook-like tip in glochidia of massulae
Azolla	*A. filiculoides*	Latin America	3-Floats	Hook-like tip in glochidia of massulae
Azolla	*A. rubra*	Oceania	3-Floats	Hook-like tip in glochidia of massulae
Azolla	*A. microphylla*	Latin America	3-Floats	Hook-like tip in glochidia of massulae
Azolla	*A. mexicana*	Latin America	3-Floats	Hook-like tip in glochidia of massulae
Azolla	*A. caroliniana*	Latin America	3-Floats	Hook-like tip in glochidia of massulae

2.5. Reproduction of *Azolla*:

Azolla reproduces by vegetative and sexually reproductions. Under natural conditions, *Azolla* multiply by vegetative reproduction. Under certain conditions the formation of sexual organs is observed and a new generation is formed from the fertilized embryo. Little is known about the condition for spore formation and its ecological significance (**Watanabe, 1982 and Lumpkin, 1987 a**).

Vegetative reproduction is the most common way of reproduction in nature and in agricultural application consists of multiplication by fragmentation of the fronds. This occurs when secondary rhizomes or branches form abscission layer at their bases and break off from the main rhizome. A root starts emerging from the base of the separating branch as soon as the cell of the abscission layer starts maturing. After separating from the parent plant, and the new plants behave independently. As the young plant grows in size, it also grows more roots, it is fully grown in 15-20 days (**Becking, 1978; Watanabe, 1982; Lumpkin and Plucknett, 1982 and Van Hove 1989**).

Azolla plant is a heterosporous i.e., having two kinds of spores which are produced on the same plant. The sporulation mostly occurs during a specific period of the year and not all the species (**Ashton, 1977; Becking, 1978 and Peters *et al*., 1978**).

At the first of sporulation ventral leaf lobe initial of a lateral branch, instead of forming a leaf lobe, the fern produces two sporocarps which are of two types, the microsporocarps and the megasporocarps. The microsorocarps, which are about 2mm in diameter, each produce 8-130 microsporangia, each containing 32 or 64 spores which called microspores aggregated into 3-10 massulae (**Moore, 1969**). The megasporocarps, which are about 0.5mm in diameter each produces a single megasporangium containing a single megaspore. At maturity, both the megasporocarps and microsporocarps dehisce. The microsporangia break open, releasing microspores into the filamentous structures known as glochidia and sink to the bottom. After a period of dormancy each microspore germinates and grows into a prothallus that in turn produces ciliated, male gametes (antherozoids). The megaspore, still joined to the megasporocarp, germinates and develops into a prothallus that produces female gametes (Oospheres). Oospheres are fertilized by antherozoides and form embryos. Each embryo germinates and pushes itself out of the sporocarp to form a plantlet, the sporophyte, that floats to the water surface, it takes one or two months from germination to produce a branched frond **(Watanabe, 1982)**.

El-Shahat (1997) found that spores were produced only by *Azolla pinnata*. Moreover, summer season with its long day hour, high light intensity and high temperature represents the most suitable conditions for *Azolla pinnata* sporulation compared to those of other seasons. The lowest amount of sporulation was observed in winter.

2.6. Chemical Composition of *Azolla*:

Chemical composition of *Azolla* is considered an another selective criterion which varies not only according to species and ecotypes but also with the ecological conditions and phase of growth. Various aspects of the chemical composition can be considered, depending on whether *Azolla* will be used as a green manure, food or other uses. *Azolla* plants have 94.96% percent moisture content. A range of chemical composition of *A. pinnata* was given by as follows: (percent on dry weight basis): Ash, 10.5; crude fat, 3.0-3.36; Crude protein, 23.0-30.0, Nitrogen, 4.0-5.0; Phosphorus, 0.5-0.9; Calcium, 0.1-1.0; Potassium, 2.0-4.5; Magnesium, 0.5-0.65; Manganese, 0.11-0.16; Iron, 0.06-0.26; soluble sugars, 3.4-3.5; starch, 6.5-6.54 and chlorophyll a, 0.34-0.55. *Azolla* has a favorable composition of amino acids and it

does contain all the essential ones (**Singh, 1979; Liu, 1979 and Van Hove, 1989**).

The nutrient composition of *Azolla* be affected by the time or age of harvest, manner of drying and exposure to sunlight, it has been found that the sun dried *Azolla*, has higher nitrogen value than air dried (**Alviar, 1984 and Van Hove, 1989**).

Total carbohydrate content of *Azolla* plants exposed to different dark and light conditions showed no significant differences. They proved that *A. pinnata* had higher carbohydrate levels than *A. microphylla* and *A. filiculiodes*. (**Herzalla *et al*., 2001**).

2.7. Environmental Factors Affecting *Azolla* Growth:

2.7.1. Water and Humidity:

As a delicate aquatic plant, *Azolla* can survive only for a few minutes on a dry surface under the tropical sun, and for a few days on paddy soil that dries during intermittent rains. Some varieties can survive indefinitely on moist, shaded mat, but will not multiply to any useful extent without a water surface on which *Azolla* can be spread. Thus, without good water control and availability, *Azolla* multiplication may not succeed. The optimum relative humidity needed for *Azolla* growth is 85-90%. In relative humidity less than 60% *Azolla* becomes dry and fragile in and complete dryness it dies (**Moore, 1969; Peters, 1975 and 1976; FAO, 1982; Watanabe, 1982 and Lumpkin, 1987a**).

Peters (1990), reported that the growth of *Azolla* is promoted by a fairly shallow depth of water in which there can be little turbulence. **Kushari and Watanabe (1992)** found that the availability of nutrients to *Azolla* depends largely on the amount of nutrients present in water rather than in the soil.

Hechler and Dawson (1995) proved that nitrogenase activity in *A. caroliniana* was maximum when the moisture content of the tissue was 82-95% of the fresh mass; but under moisture stress, when the moisture content dropped to 80%, nitrogenase activity decreased to less than one – fifth of its maximum.

2.7.2. Light and photoperiods:

The Eukaryotic *Azolla* and prokaryotic *Anabaena* are both photosynthetic organisms and their pigmentation is complementary. The *Azolla* contains chlorophyll a and b as well as carotenoids and phycobiliproteins. Nitrogen fixation was already saturated at low light intensity in the intact association of *Azolla* (**Peters and Mayne, 1974 a and Peters, 1976**).

The growth rate of *Azolla* has been reported to saturate at 25-50% of full sunlight and is not inhibited by full sunlight as long as other factors are not limiting (**Ashton, 1974; Basavana *et al*., 1980 and Talley and Rains, 1980a**).

Daylength is another important aspect of light. Growth rate has been shown to positively correlate to daylength and continues to increase up continuous illumination (**Peters *et al*., 1980 b and Lumpkin and Bartholomew, 1986**).

Azolla spp grow best in less than full sunlight except in light latitudes during spring. Growth and nitrogen activity were the highest at 50% of full sunlight (**Lumpkin and Plucknett, 1980).** Light intensity and temperature prevailing during the hot season suppressed *Azolla* growth on the other hand, low light intensities under a dense growth of rice cause *Azolla* to suffer or die (**Lumpkin, 1987a and Kondo *et al*., 1989**).

2.7.3. Temperature:

The optimum temperature for *Azolla* spp. is between 18 and 28°C (**Tuan and Thuyet, 1979**), but some species can survive a very wide temperature as *A. pinnata, A. mexicano*, and *A. Caroliniana* Although growth rates are reduced above 35 °C and no species can survive at prolonged temperature above 45 °C (**Lumpkin, 1987a**).

El-Haddad *et al*. (1988a) suggested that the optimum climatic conditions for maximum growth of *A. pinnata* is prevailing in summer. The highest value of nitrogenase activity was obtained in August at the end of 3rd week of incubation being 13.8 μmol C_2H_4/g dry wt hr^{-1} in case of *A. pinnata*. **Peters (1991)** reported that an Australian isolate of *A. pinnata* could survive at temperature of 55 °C.

Hechler and Dawson (1995) found that nitrogenase activity in *A. caroliniana* was very low early in the morning, rose sharply during midmorning attained a maximum at 12:00 – 3:00 p.m. and then fell to a low level in the evening. Also, nitrogenase activity in *Azolla caroliniana* could be detected at temperature between 5 and 40 °C, it was relatively high between 15 and 35.

2.7.4. Hydrogen Ion Concentration (pH):

The response of *Azolla* to pH is greatly affected by other factors of the environment such as light intensity, temperature, and soluble iron. Whereas, the optimum growth of *Azolla* in culture solution is in pH range of 4.5-7, but *Azolla* can survive in pH 3.5-10 if all essential elements are available (**Nickell, 1961 and Ashton, 1974**).

Watanabe *et al*. (1977) reported that *Azolla* growth was decreased by increasing pH level at low iron concentration. Where, the growth and N-accumulation were lowest at pH.7.5 in the iron- deficient medium. Meanwhile pH higher than 6.5, *Azalla* fronds turned yellowish in the same iron – deficient medium.

At high light intensity optimum pH is 9-10. Whereas at low light intensity, optimum pH is 5-6 (**Tuan and Thuyet, 1979; Lumpkin and Plucknett, 1980 and**

Hamdi, 1982).

Lumpkin and Plucknett (1982) reported that, alkaline and excessively limed soils will resulted in reducing phosphate availability because of the precipitation of calcium and magnesium. The availability of most micronutrients to *Azolla* plant increases with decreasing pH. Also the increasing solubility of aluminum, iron and manganese with increasing acidity may cause toxicity and may interfere with the absorption of Ca, Mg and other basic cations. In contrast to the most other micronutrients, Mo-deficiency may occur from decreasing in pH.

El-Haddad *et al*. (1988) proved that pH 3 unsuitable for growth of *A. pinnata* and *A. filiculoides* as they failed to grow at this pH. But *A. pinnata* was more resistant to pH changes than *A. filiculoides*. They also found that the maximum dry matter content of both *Azolla* was obtained at pH 8.

In greenhouse experiments, **Wagner (1997)** found that at water temperature of 25 °C, both *A. pinnata* and *A. filiculoides* showed maximum growth at pH values from 5-7. On the other hand, *Azolla pinnata* showed greater tolerance to a wide pH range than did *A. filiculoides* the latter growing much more poorly at pH values of 4 and 8. Meanwhile, **Dawar and Singh (2001)** reported that N fixation decreased at neutral pH.

2.7.5. Mineral Nutrition:

Like most plant, *Azolla* is sensitive to change deficiencies in the supply of plant nutrients. For optimal growth, the fern requires all the macro and micronutrients elements which are essential for normal plant growth, **Moore (1969).**

Yatazawa *et al*. (1980) determined that, the threshold levels of P, K, Mg and Ca required in the medium for *Azolla* growth were approximately 0.03, 0.4, 0.4 and 0.5 $mmoll^{-1}$, respectively. Whereas full nitrogenase activity required 0.03, 0.6, 0.5 and 0.5 $mmoll^{-1}$ respectively. On the other hand the threshold levels of the micronutrients Fe, Mn, Mo and B, for *Azolla* growth, were 50, 20, 0.3 and 30 μgl^{-1}, respectively. Meanwhile macronutrients such as N.P.K. Ca and Mg are specially important and manifast marked effect on growth of the fern if present in too high or too low concentration.

Normally, nutrients must be available in the water, though in very shallow water, *Azolla* may extract some nutrients from the soil. Moreover, adequate nutrient levels should be maintained throughout the period of growth, **Tung and Shen (1985).**

1.1.1 2.7.5.1. Macronutrients:

2.7.5.1.1 Nitrogen:

The application of combined nitrogen to the medium will alter nitrogen

metabolism in the *Azolla-Anabeana* complex, but its effect on nitrogen fixation is expected to differ from that in free living *Anabaena*, since symbiotic *Anabaena* is located in the leaf cavities of the host and has no direct contact with the outside environment. (**Bone, 1971; Ohmori and Hattori; 1972 and 1974**).

Free living blue-green algae and *Azolla* can grow on a medium containing ammonium or nitrate as well as on a nitrogen – free medium. Algal symbionts separated from the host plant were more insensitive to ammonia than free living *Anabaena* (**Ohmori and Hattori, 1972 and Peters, 1975**).

Nitrogen fixation in *Azolla* was completely inhibited by 10 mM ammonium and 25 mM nitrate at 4 days after the start of the incubation. But nitrate was absorbed by roots and translocated to shoots considerably more slowly than was ammonium (**Ito and Watanabe, 1983**).

El-Haddad *et al*. (1988b) found that application of 40 ppm urea-N secured the highest growth yield for both *A. pinnata* and *A. filiculodies*. Whereas in the presence of ammonical-N the highest growth yield was secured at 40 ppm and 10 ppm for *A. pinnata* and *A. filiculodes*, respectively. The nitrogen content of *A. pinnata* was stimulated by increasing ammonium sulfate or urea up to 40 ppm but *A. filiculodes* was stimulated effect at 10 ppm of ammonium sulfate only. **Singh and Singh (1989)** found that the application of nitrogen reduced heterocyst frequency in *Anabaena azollae*.

Hechler and Dawson (1995) found that the source of nitrogen affected the response of *A. caroliniana*. Whereas 10.0 mg NO_3-N^{-1} increased growth rate, the same concentration of ammonium – nitrogen decreased growth rate and urea had no effect. Also they proved that 2.0 mg NH_4-NI^{-1} was sufficient to cause a significant reduction in nitrogenase activity in *A. caroliniana*, whereas 10.0 mg NO_3-NI^{-1} increased nitrogenase activity by 50%.

2.7.5.1.2. Phosphorus:

Phosphorus is one of the most important and often limiting nutrient for *Azolla* growth. Phosphorus deficiency is indicated by smaller, less vigorous plants and may causes the plants to become pink to deep red and fragile and to develop very long roots. Phosphorus concentration less than 0.6 ppm in the nutrient solution was found to decrease growth rate, nitrogen fixation and the chlorophyll content of *A. pinnata* (**Cohn and Renlund, 1953 and Subudhi and Watanabe, 1979**).

Yatazawa *et al.* (1980) found that the presence of phosphate in the nutrient solution less than 0.03 mmol/L causes deficiency in growth rate and nitrogen fixation in *Azolla*.

Watanabe and Ramirez (1984) concluded that *A. pinnata* can grow satisfactorily without phosphorus application in the soils with Olsen phosphorus value higher than 30 mg kg^{-1} and phosphorus absorption capacity lower than 1500 mg P_2O_5/100 g^{-1} soil. Also they observed that a great variation in *Azolla* growth among different soils with similar contents of available phosphate. They suggest that factors other than phosphate may affect the *Azolla* growth. Meanwhile, **Watanabe and Espinas (1985)** reported that 25 ppm P in the soil is optimum for *Azolla* growth.

Phosphorus content of *Azolla* grown in pots with different soils and in the field showed that the threshold value of phosphate deficiency was 0.4% P in *Azolla* (on dry weight basis), 20 ppm P in soil, and 0.15 ppm P in flooded fields (**Watanabe and Ramirez, 1984, Ali and Watananbe, 1986 and Watanabe *et al*., 1986**).

1.1.2 2.7.5.2. Micronutrients:

Micronutrients such as molybdenum (an essential constituents of nitrogenase), Cobalt and iron are required for nitrogen fixation (**Peters, 1991**).

2.7.5.2.1. Iron:

Iron is a common limiting element, since it is an essential constituent of nitrogenase (**Fogg *et al*., 1973**). **Watanabe and Espinas, (1976)** reported that, 1 ppm Fe was sufficient for rapid growth of *Azolla*. The critical concentration of iron for *Azolla* growth was 20-50 μg FeI^{-1}. Whereas Ferric ion were so readily available at pH 4 that a high concentration of calcium was required to balance the increased absorption of iron. Otherwise the fronds suffered from iron toxicity (**Lumpkin and Plucknett, 1980**). At the same manner, iron deficient plants become yellow due to the depletion of chlorophyll. Roots become thin and whitish (**Malavolta *et al*., 1981**). The availability of iron is decreased by neutral to alkaline conditions (**Lumpkin and Plucknett, 1980 and Watanabe, 1982**).

A. filiculoids required iron for growth and N_2-fixation which increased at level up to 5 mg FeI^{-1} as Fe-EDTA. Iron deficiency problems are aggravated in high pH condition since ferric ions are precipitated and become unavailable for plant absorption (**Singh *et al*., 1984 b and Wagner, 1997**).

2.7.5.2.2. Cobalt:

Cobalt is required for nitrogen fixation and the symbiotic growth of *Anabaena azollae* in the host plant. In the absence of combined nitrogen, *Azolla* growth, chlorophyll content and nitrogen fixation is increased by the addition of cobalt (**Johnson *et al*., 1966 and Lumpkin, 1987b**).

2.7.6. Salinity:

Rajrathinam and Padhya, (1989) reported that *A. filiculoides* can tolerate salinity up to 20 mM NaCl but *A. pinnata* can tolerat 40 mM NaCl in the medium. Increasing the concentration of NaCl to 40 and 50 mM strongly inhibited the growth of *Azolla* (fresh and dry weight). *Azolla* was killed within two weeks in presence of 50 mM NaCl.

The nitrogen content of *Azolla* fronds decreased gradually by increasing the salt concentrations up to 40 and 50 mM NaCl but at 85 mM NaCl was toxic to *Azolla* sp. (**Tantawy and Herazalla, 2003**).

2.8. *Azolla* Utilizatiom:

Azolla-Anabeana has many uses. It can be utilized as a biofertilizer for rice and other crops, including taro, wheat and many others, an animal feed, a human food , medicine, and water purifier. It may also be used for the production of hydrogen fuel, the production of biogas, the control of weeds, the control of mosquitoes, and the reduction of ammonia volatilization that accompanies the application of chemical nitrogen fertilizer (**Wagner, 1997**).

2.8.1. *Azolla* as Biofertilizer:

The utilization of biofertilizers has several advantages over chemical fertilizers:

I. Biofertilizers are inexpensive, making use of freely available solar energy, atmospheric nitrogen and water.

II. Biofertilizers utilize renewable resources, whereas the production of chemical fertilizers depends on petroleum, admonishing resource.

III. Biofertilizers are non polluting.

IV. Besides supplying nitrogen to crops, biofertilizers also supply other nutrients vitamins and growth substances.

V. Biofertilizers improve the general fertility of the soil by increasing the availability of number of nutrients to crops by due to the increasing the organic matter in the soil, and improving soil structure (**Venkataraman and Kaushik, 1980**).

1.1.3 2.8.1.1. Rice Production:

The use of chemical fertilizers in rice fields is expensive, disturbs the equilibrium of agroecosystems, and causes pollution of the environment. These problems may be avoided by the use of biofertilizers. For many centuries, *Azolla* has been used to successfully increase rice yield in Vietnam and Southern China (**Fogg**

***et al*., 1973; Madhusoodanan and Sevichan, 1992 and Watanabe and Liu, 1992**).

Singh *et al*. (1992) compared the effectiveness of *Azolla* with other types of biofertilizers, in a study on the effects of free- living blue-green algae, *Azolla* and chemical nitrogenous fertilizers, rice grain yield was the highest with the application of *Azolla* + 120 kg N ha^{-1} (5.01 t ha^{-1}), followed by blue green algae + *Azolla* (4.62 t ha^{-1}) + 60 kg N ha^{-1} and, lastly, by 120 kg N ha^{-1} (4.61 t ha^{-1}). In Egypt, **El-Shahat, (1997)** found that, application of 40 or 60 kg N derived from *Azolla*, generally stimulated growth and yield of three varieties of rice. This effect was shown in the number of panicle, weight of 1000 grains, grain and straw yields and N% in grain and straw.

El-Shahat *et al*. (2002) found that in rice production the highest plant height, panicle length, panicle weight, 1000–grains weight, grain and straw yields and crude protein were obtained from the biochemical treatment of (30 kg N/fed + *Azolla*).

Herzalla *et al*. (2003) found that *Azolla* significantly increased rice yield and soil organic carbon. Sixty kg N fed^{-1} as *Azolla* was almost equivalent the application of 60 kg N fed^{-1} as urea. The combination of urea and *Azolla* 30 kg N fed^{-1} each resulted in grain yield higher than that obtained with urea or *Azolla* alone but not significantly different from that obtained with 60 kg N fed^{-1} as urea. The soil organic carbon increased over the control by 28% for *Azolla* treatment, 41.1% for urea – *Azolla* combination and no change with urea was observed.

1.1.4 2.8.1.2. Wheat Production:

Mahapatra and Sharma (1989) found that, the application of *Azolla* with Sesbania had beneficial residual effects on subsequent wheat crops, raising grain yield by 56-69% over controls.

Azolla also was beneficial to wheat when applied in a rooting rice- wheat cropping system. *Azolla* applied as a monocrop between the wheat and rice crops, or applied as intercrop with rice, has a significant beneficial effect on subsequent wheat crops (**Kolhe and Mittra, 1990**).

Marwaha *et al*. (1992) found that, the application of *Azolla* increased grain yield of wheat, though straw yield and the number of tillers per plant were largely unaffected.

Adel *et al*. (2000) found that, a significant increases were occurred for all the studied parameters (such as grain yield, spike length and straw yield) as a result of applying the nitrogen rates up to 75 kg N fed^{-1}. Also, all the studied parameters were significantly increased due to the applied *Azolla* up to 50 kg N fed^{-1}., except of grain yield, spike length and 1000- grain weight up to 25 kg N fed^{-1}. The treatment of grain

coating by micronutrients showed a noticeable increment in all the studied parameters. The highest grain yield and crude proteins were obtained from the treatments of grain coating (75 kg N fed^{-1}) and *Azolla* (25 kg N fed^{-1}). While, the highest yield of both straw and micronutrients uptake was obtained from grain coating (75 kg N fed^{-1}) and *Azolla* (50 kg N fed^{-1}).

1.1.5 2.8.1.3. Tomato Production:

Selim *et al*. (1999) concluded that, *Azolla* incorporation singly proved to be the most effective treatment for obtaining high growth and yield of tomato plants without the addition of inorganic N-fertilizers. So, it can be recommended to incorporate *A. pinnata* in soil as a green manure 15 days before tomato cultivation. Inorganic N-fertilizer can be totally substituted by *Azolla* application, and consequently such practice will reduce environmental pollution, caused by continuous application of chemical fertilizers, will reduce agricultural costs of tomato cultivation and will increase tomato yield to significant extents.

El-Shahat *et al*. (2000) studied that, the effect of *Azolla* pinnata, as a green manure, other traditional organic and inorganic manures (Farm-yard manure “FYM”, compost and ammonium nitrate) on the growth and yield of tomato plants cultivated in sandy soil. The obtained results showed that *Azolla* incorporation remarkably increased the growth and NPK contents of tomato plants, in comparison to FYM, compost or ammonium nitrate amended plants. Number and weight of tomato fruits were significantly higher than those obtained in case of soil supplementation with FYM, compost or ammonium nitrate.

1.1.6 2.8.1.4. Other Crops:

In the case of permanent crops such as bananas, *Azolla* is applied as a mulch on the soil surface around the bases of the plants. When there is an overproduction of *Azolla*, it can be mixed with soil and rice straw to form compost. Super phosphate may be added to reduce nitrogen loss (**Van Hove, 1989**).

1.1.7 2.8.2. Soil Fertility:

Most of the nitrogen fixed becomes available to rice only after the *Azolla* has decomposed, although a small amount of ammonium is released into the water by *Azolla* during growth (**Moore, 1969; Silvester, 1977; National Academy of Sciences, 1979 and Watanabe, 1984).**

Chung-Chu (1984) determined that 3-4% of the total nitrogen fixed by *Azolla* is excreted into the medium during its growth.

Van Hove (1989) found that, when *Azolla* incorporated to soil, improves soil structure because of its high productivity, which supplies large quantities of organic matter. *Azolla* decomposes rapidly, and therefore the nitrogen has fixed, the phosphorus and other nutrients may be absorbed from the water, perhaps in competition with the rice, are rapidly released back into the medium and made available for uptake by rice during grain development.

Singh and Singh (1990a) found that *Azolla* application improves soil fertility by increasing total nitrogen, organic carbon, and available phosphorus in the soil. These findings are supported by **Satapthy (1993) and Thangaraju and Kannaiyan (1993**), who found that the most effective application for increasing soil fertility was first culturing *Azolla* as a monocrop, incorporating it before transplanting, and subsequently culturing it as an intercrop with two incorporations.

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

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

Selim *et al*. (1999) found that the growth of most of soil organisms (*Azotobacters, Azospirilla*, fungi, and total microbes) considerably stimulated. Microbial biomass gradually increased after the application of various kinds of organic matter, among them *Azolla* is the most common especially in tropical areas. Increasing the growth of different microbial groups in soil as a result of *Azolla* amendment may be due to its high content of readily available carbon content and the production of growth factors.

2.8.3. Other Uses:

1.1.8 2.8.3.1. Animal Feed:

Sculthorpe (1967) reported that *Azolla* is harvested in large quantities from water bodies in parts of tropical Africa, India, and South East Asia and utilized as fodder for cattle and pigs.

Azolla has been abundantly described as food for various animals (fish, cattle, sheep, goats, rabbits and geese) and has actually been widely used especially for pigs, fish and ducks in China and Vietnam. (**Sanagina and Van Hove, 1989**) showed that *Azolla* is indeed rich in protein and has a relatively good amino acid balance; more quantitative information would nevertheless be useful on the nutritive value, as fresh, dried or ensiled material, on the digestibility and on the palatability of various *Azolla* species and strains.

Das *et al*. (1994) found that digested *Azolla pinnata* slurry remaining after biogas production was suitable as a fish pond fertilizer, which, significantly increased phytoplankton populations in comparison with either digested *Azolla pinnata* slurry or row cow dung.

Azolla has been used as food for hens. *Azolla* meal replace sesame meal on a digestible protein and digestible amino acid basis up to 200 g kg^{-1} diet of lying hens but only to 150g kg^{-1} diet when the diet is formulated on a total protein and total amino acid basis. Feeding *Azolla* on a digestible protein and digestible amino acid basis maintained or improved protein efficiency. Feeding *Azolla* on a total or digestible nutrient basis had no effect on egg quality except egg size and yolk color. Egg size improved when the diets were formulated on a digestible protein and digestible amino acid basis. Yolk color was significantly improved with increasing levels of *Azolla* meal and longer feeding. Feeding *Azolla* meal on a digestible protein and digestible amino acid basis produced better egg production than the control diet or diets formulated on a total protein and total amino acid basis. Hen-day egg production with 50g kg^{-1} *Azolla* meal using digestible protein and digestible amino acid was improved by 6.4 and 9.9% over the control diet and the diet with 50 g kg^{-1} *Azolla* meal on digestible protein and digestible amino acid egg production improved by 2.0 and 5.7% over the control diet and 100 g kg^{-1} *Azolla* meal on total protein and total amino acid (**Khatun *et al*., 1999**).

1.1.9 2.8.3.2. Human Uses:

Azolla appears to be fit for human consumption. Few researches have experimented with the preparation of *Azolla* in soup or "*Azolla* – meat balls" as food for man (**Van Hove, 1989**).

1.1.10 2.8.3.3. The Control of Weeds:

Krock *et al*. (1991) found that a coverage of *Azolla* reduced the total amount of weeds. Also, *Azolla* cover reduced light intensity be about 90%, reducing photosynthesis in the floodwater and thus reducing oxygen concentration of the water by more than 50%. Besides reducing light intensity, *Azolla* cover laters light quality, the green leaves having a filter effect that increases the relative amount infrared rays.

1.1.11 2.8.3.4. Control of Mosquitoes:

For a thick *Azolla* mat on the water surface can prevent breeding and adult emergence. In survey of pools, ponds, wells, rice fields, and drains, breeding by *Anophetes* spp. was almost completely suppressed in water bodies that were completely covered with *Azolla* (**Ansari and Sharma, 1991 and Wagner, 1997**).

1.1.12 2.8.3.5. The Water Purification:

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

3-MATERIALS AND METHODS

3.1. Materials:

3.1.1. *Azolla* Strain:

Azolla pinnata used in the present study is illustrated in picture (1). The *Azolla* kindly supplied by Soils, Water and Environment Research Institute (SWERI) from the International Rice Research Institute (IRRI), Los Banos, Lagona, Philippines.

3.1.2. Standard Inocula:

The collected *Azolla* surface was sterilized with a solution of mercuric chloride 0.1% for 30S according to **Vandna and Ashwani, (1998)** washed by sterilized water for several times and then air dried on tissue papers for 30 minutes. One g fresh *Azolla* per plastic dish (14 cm diameter and 7cm depth) was used as standard inoculum in all experiments. Five replicates for each treatment were carried out. The inoculated dishes were kept under greenhouse conditions during the studied period.

3.1.3. Soil Used:

A sample of soil was collected from Kaliobia Governorate to be used for propagation of *Azolla* in nursery, and to study the effect of *Azolla* on growth and yield of wheat cultivated in pots. The collected soil were analyzed for their chemical and physical properties according to **Page *et al*., (1982)** in the Dept. of Plant Nutrition, Soils, Water and Environment Res. Inst. (SWER); Agric. Res. Center (ARC), Giza, Egypt. The chemical and physical properties of the tested soil was present in Table (2).

Picture (1) : *Azolla pinnata* fronds

Table (2): Physical and Chemical analysis of experimental Soil.

Mechanical analysis		Chemica analysisl							Physical analysis	
Coarse Sand	13.90	Soluble cations (meq/100 g soil)				Soluble anoin (meq/100 g soil)			WHC %	75.50
Fine sand %	9.10								$CaCO_3$ %	3.14
Silt %	25.30	Ca^{++}	Mg^{++}	Na^{++}	K^{+}	HCO_3^{-}	Cl^{-}	SO_4^{--}	O.M %	1.10
									E.C.	1.50
Clay %	51.70								Total N %	0.31
Soil texture	Clay	3.90	7.10	5.80	4.70	3.70	10.80	7.00	Soil pH	7.70

E.C.: Electrical conductivity (dsm).

O.M.: Organic Matter %

WHC: Water holding capacity %

3.1.4. Meida Used:

Different media were used in this study as growth media for *A. pinnata.*

3.1.4.1. Yoshida Medium:

(**Yoshida *et al.*, 1976**). This medium was prepared using the following chemical composition in ppm:

a- <u>Macroelements:</u>

$NaH_2PO_4.2H_2O$	40.00
K_2SO_4	40.00
$CaCl_2.H_2O$	40.00
$MgSO_4.7H_2O$	40.00

b- <u>Microelements:</u>

$MnCl_2$-$2H_2O$	0.50
$NaMoO_3.2H_2O$	0.15
H_3BO_3	0.01
$ZnSO_4.7H_2O$	0.01
$CuSO_4.5H_2O$	0.01
Fe-EDTA	2.00
PH	5.50

3.1.4.2. Soil Medium:

Special weight of soil (200 g) was added to 500 ml tap water for every plastic dish was used.

1.1.13 3.1.4.3. Van Hove Medium (Van Hove *et al.*, 1983):

This medium was prepared as:

KH_2PO_4	54.40 mg
$CaCl_2.2H_2O$	294.00 mg
$MgSO_4.7H_2O$	192.00 mg
KCl	150.00 mg
NaCl	12.00 mg
Fe(EDTA)	12.00 mg
Trace metal	1.00 ml

Micro element stock solution: (0.50 ppm $MnCl_2.2H_2O$, 0.15 ppm $NaMoO_3.2H_2O$, 0.10 ppm H_3BO_3, 0.01 ppm $ZnSO_4.7H_2O$ and 0.01 ppm $CuSO_4.5H_2O$). In this medium 1 ml of trace metal was mixed with 4.3 mg $CoCl_2.6H_2O$.

3.2. Experimental Techniques:

3.2.1. Propagation Of *Azolla* In Nursery:

Azolla growing in plastic dishes of 35 cm in diameter and 15 cm depth containing 1.5 kg soil and 3 liters of tap water. Dishes were kept in a greenhouse till *Azolla* covered the entire surface of the water then it was harvested and washed gently in running tap water for several times by using screen of 0.2 mesh and air dried on tissue paper according to **El-Shahat (1988).**

3.2.2. Selection of The Most Active Medium for the Growth of *Azolla*:

In this experiment *Azolla* was cultivated on three different media (Yoshida, Soil and Van Hove) to select the best medium having the highest growth rate and N_2-fixation efficiency of *Azolla* to be used in subsequent studies.

3.2.3.Effect of Different Concentrations of Zinc and Cobalt on Growth, N_2-Fixation and Nutrients Uptake of *A. Pinnata*:

In this purpose, modified yoshida medium (**Yoshida *et al*. 1976**) was supplemented with different concentrations of Zn (0, 5, 10, 15, 20, 25 and 30 ppm) as EDTA-Zinc and Co (0, 1, 2, 3, 4 and 5 ppm) as $CoCl_2$. The medium was distributed in plastic dishes (14 cm diameter x 7cm depth) each dish was filled with 600 ml of Yoshida medium. The *Azolla pinnata* fronds were grown for one week on control Yoshida medium applied at a rate of one g $dish^{-1}$. which was used as a standard inocula in these experiments. Five replicates were carried out for each treatment. The inoculated dishes were incubated under greenhouse conditions. In the culture of Zn and Co, the Yoshida medium was changed every five days to get constant pH value and concentration of the elements through out the experiments time. The *Azolla* medium in different treatments was kept at a constant volume through out the experimental periods by compensating the evaporated water by adding deionized water when it is necessary. Developed *Azolla* growth was periodically sampled at 5, 10, 15, 20 and 25 days to determine fresh and dry weights, nitrogenase activity, total nitrogen, phosphorus, potassium, zinc and cobalt contents.

3.2.4. The potential of *Azolla* Nitrogen as Limited Factor For Wheat Production:

A pot experiment was carried out in greenhouse in soils, water and Environment Res. Inst. Giza, Egypt during the period from November 2002 to April 2003, to study the effect of *Azolla* as dry manure and urea application on growth and yield of three varieties of wheat (Giza 168, Gemiza 9 and Sakha 93). The nitrogen fertilization rate recommended by Ministry of Agriculture and land Reclamation (75 kg N fed^{-1}). The experiment was conduced in pots with 40cm in diameter and 40cm in depth which filled with 10 kg soil pot^{-1}. Pots were filled with soil and mixed with superphosphate (100 kg fed^{-1}). Air dried *Azolla* materials was applied one week before wheat seedlings (three seedlings/pot) were developed. *Azolla* is used as nitrogen source on wheat yield by using different levels of *Azolla pinnata* incorporation alone or mixed with chemical nitrogen fertilizer (urea 46% N) in different doses to give a recommended dose finally. Also, urea is used alone as nitrogen source which is considered as control. The design of experiment was complete with four treatments and five replicates for each variety as the following:

Treatment No.	Kg.N. as urea/ fed	Kg N as *Azolla* fed^{-1}
T1	75	0

T2	50	25
T3	25	50
T4	0	75

Urea dose was added in two equal split doses, i.e., one at 20 days after seedling and the other at the trillering stage. Pots were kept under greenhouse conditions for maturity stage.

At harvest , wheat yield components such as plant height (cm), panicle weight (g pot^{-1}), straw yield (g pot^{-1}), grain yield (g $polt^{-1}$), 1000-grain weight (g) and harvest index were determined. As well as the nitrogen percentage for both grain and straw ere recorded.

After wheat harvesting, the remained soil in pots was subjected for chemical and physical analysis according to **Jakson (1973).**

3.3. *Azolla* Determinations:

3.3.1. Fresh Weight:

Azolla fronds were harvested washed with deionized water and placed under shade between two thick layers of blotting tissue papers for approximately 1-2 hours before determining fresh weight. Fresh weight of *Azolla* fronds was expressed as g m^{-2}.

3.3.2. Dry Weight:

Dry weight of *Azolla* was determined by drying fronds at 70 °C to constant weight. Dry weight of *Azolla* was expressed as g/m^2.

3.3.3. Doubling Time:

Growth rate of *Azolla* in terms of doubling time (D.T.) was calculated by using the following equation according to **Aziz and Watanabe (1983)**:

Doubling time = t/r whereas:

t=the duration of *Azolla* growth

r = [log(wt/wo)/0.301]

Wt= weight of *Azolla* at time t

Wo= weight of *Azolla* at Zero time, i.e. weight of inoculum.

3.3.4. Nitrogen Determination:

Nitrogen content of *Azolla* fronds was determined in the dried plant materials by microkjedahl method according to **Black *et al*., (1965)**. The results are presented as g N/m^2 on dry weight basis.

3.3.5. phosphorus content:

Phosphorus content of *Azolla* fronds was determined in the dried plant materials by spectrophotometer according to **Olsen and Sommers (1982)**. The results were presented as ppm on dry weight basis.

3.3.6. Potassium Content:

It was determined in plant samples by digestion of dried material with concentrated sulphuric acid and concentrated perchloric acid. The acid digests of different samples which were measured photometerically, according to **Brown and Lilliland (1946).**

3.3.7. Zinc and Cobalt Determination:

Zinc and Co were determined by atomic absorption according to **Jackson (1973)**.

3.3.8. Nitrogenase Assay:

Nitrogenase activity of *Azolla* was assayed by using the actylene reduction technique as recommended by **Hardy *et al*., (1973)**. It can be summarized as follows:

Samples of *Azolla* were placed into 500 ml capacity serum bottle. The bottles were sealed with silicon rubber stoppers, the acetylene was injected at a rate of 10% of the gas phase of the bottle volume. Bottles were incubated under the greenhouse conditions for one or two hours. Two ml of gas sample were drawn and assayed for ethylene, using a Perkin Elmer Gas Liquid Chromatography (GLC), Model 392013, filled with dual flame ionization detectors, and 1.5 m x 4mm stainless steel column, packed with Porapak N (100 mesh). Nitrogen (N_2) and air were used for the flame at rates of 30 and 300 ml min^{-1}; respectively. The detector temperature was 60 and 120 °C, respectively. Standard curve was run concomitantly to the analyzed samples. Ethylene mixture was made by injecting 1.2 ml pure ethylene (C_2H_4) into a 1200 ml serum bottle (conc. 100 ppm). Out of which serial dilutions were made.

Two ml gas of each dilution were injected into gas chromatography at several attenuations. A linear relation between ethylene concentration and ethylene peak height was obtained for each attenuation.

For calculation of ethylene amount in gas samples, the peak heights were measured in cm, and converted to the equivalent C_2H_4 using factor derived from the standard curve. The concentrations of ethylene in the samples (µl C_2H_4 values) were then converted to µmoles by dividing their value by the volume of the molecular weight of gas (22.41). The results are presented as µ mole C_2H_4 g^{-1}. dry weight hr^{-1}.

3.4. Wheat Determinations:

3.4.1. Morphological Parameters of Wheat Plant:

At maturity stage morphological characteristics of each variety of wheat under different fertilizer treatments were recorded. These characteristics were: plant height (cm), number of spikes (plant^{-1}), Grain yield (g pot^{-1}), 1000 grain weight (g) and Harvest index i.e. [(kg grain yield/ Kg grain + Kg straw yield) X 100] according to Yoshida and Parao (1972)**.**

3.4.2. Nitrogen Content:

Total nitrogen content of grain and straw was determined according to Black *et al*., (1965)**.**

3.4.3. Statistical analysis:

Wheat yield data of pot experiment were subjected to statistical analysis according to Snedecor and Cochran (1969)**.**

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6- SUMMARY AND CONCLUSION

Azolla has many uses, it can be utilized as a biofertilizer on rice and many other crops and water purifier. *Azolla* can accumulate more than 10 kg N/ha./day, and thus has the potential of supplying the entire nitrogen requirement for high-yielding many crops within a few weeks due to its rapid multiplication and N_2-fixation. *Azolla* as organic matter can also improve physical and chemical properties of the soil.

Although *Azolla* has drawn the attention of Egyptian researchers as a potential biofertilizer for rice culture and as an organic matter for other crops some aspects affecting the potential performance of *Azolla* are still in need to be studied.

This study can summarized as follows:

Azolla pinnata was cultivated in three different media viz. Yoshida, soil and Van Hove, to select the most appropriate one which secure the highest growth parameters of A. *pinnata*. Results showed that:

The highest growth yield (fresh and dry weights) when *A. pinnata* grown in Yoshida medium after 30 days of incubation, but doubling time was particularly lower in this medium after 5 days of incubation. It could be noticed that the *A. pinnata* fronds exerted higher N_2-fixing capacity in Yoshida medium compared with Soil and Van Hove media.

The nitrogen percentages and contents of *A. pinnata* were generally increased by increasing the incubation period in all different growth media, while Yoshida medium induced the highest values of nitrogen percentage and content compared with Soil and Van Hove media. Also phosphorus, potassium and zinc contents increased with increasing the period of incubation when *A. pinnata* was grown in Yoshida media. Wherever the Van Hove and Soil media recorded the same results approximately.

The second experiment was carried out to study the effect of different concentrations of zinc (0, 5, 10, 15, 20, 25 and 30 ppm-Zn) on *A. pinnata* parameters as fresh and dry weights, doubling time, nitrogenase activity and mineral composition. Results showed that: the growth decreased by increasing of zinc concentration. Doubling time generally increased by increasing concentration of zinc. *A. pinnata* after 15 days from incubation at the concentrations 10, 15, 20 ppm-Zn did not show detectable differences in doubling time. The nitrogenase activity of *A. pinnata* was not constant and exhibited fluctuated pattern during the incubation

periods. The highest N_2-fixing capacity was observed, when *A. pinnata* was grown in culture medium supplemented with 5 ppm-Zn at 5 days from incubation, being 38.64 μmole C_2H_4. g dwt^{-1} hr^{-1}. Nitrogen contents showed the highest records at the control treatment after 25 days of incubation, but, the nitrogen percentage showed the highest records with the application of 15 ppm Zn on the 25^{th} day of incubation.

Generally, zinc content increased by increasing incubation periods and zinc concentrations. The greatest value of zinc content was observed when *Azolla* fronds were grown in presence of 30 ppm-Zn after 15 days of incubation. On the other hand, the highest Zinc uptake, recorded particularly at concentration of 20 ppm-Zn after 25 days of incubation. Phosphorus content generally increased by increasing the concentration up to 15 ppm-Zn and then at higher concentrations (20, 25 and 30 ppm) phosphorus content decreased. The highest values of phosphorus and potassium contents were reported at Zinc concentration of 15 ppm after 25 days of incubation.

The third experiment was carried to investigate the effect of different cobalt concentrations of (0.0, 1, 2, 3, 4 and 5 ppm) on the growth parameters when *A. pinnata* was grown on Yoshida medium. The results showed that: the growth of *A. pinnata* in terms of fresh and dry weights increased by increasing the cobalt concentration up to 2 ppm *A. pinnata* appeared to be sensitive to high concentrations of cobalt. Doubling time of *A. pinnata* was particularly lower at concentration of 2 ppm-Co. At concentrations above 2 ppm-Co, the doubling time was gradually increased up to the highest levels of cobalt. The highest record of acetylene reduction was generally reported at 5 ppm-Co after 5 days of incubation. The nitrogen percentages and contents of *A. pinnata* increased in fronds by increasing the incubation period. The highest nitrogen percentages and contents were obtained at the concentration of 2 ppm Co.

Regarding to, phosphorus, potassium and zinc contents of *Azolla* the highest records for *A. pinnata* were obtained with the concentration 2 ppm-Co after 25 days of incubation. The same concentration of cobalt enhanced, both cobalt content and cobalt uptake by *A. pinnata* specially after 20 and 25 days of incubation. Lastly, the application of 2 ppm-Co stimulated growth, N_2-ase activity, N-P-K- and Zn contents, as well as cobalt content and cobalt uptake by *A. pinnata* grown in Yoshida medium.

In pot experiment five replicates were carried out to evaluate the effect of *A. pinnata* and/or urea application on growth and yield of three wheat varieties, Giza 168, Sakha 93 and Gemiza 9. Results could be summarized as following:

- Application of 75 kg N-*Azolla* generally stimulated grain yield of the three varieties of wheat non significantly.

- The combination of urea and *Azolla* had less favorable measured parameters than *Azolla* alone.
- The application of *Azolla* alone led to increase the nitrogen percentage of grains significantly .
- Mixing *Azolla* with 50 kg N/fed urea gave favorable results than those of mixing with 25 kg N/ Fed – urea.

The soil organic matter content after wheat harvesting was significantly increased over the initial soil treatment (control) due to the residual effect of either *Azolla* or urea applied each alone or in combination at different rates during wheat cultivation. *Azolla* application had improved soil fertility by increasing total nitrogen.

We can conclude from the present study the following:

1- Yoshida media secured the best results on *Azolla* growth parameters such as growth rate, nitrogen accumulation and nitrogenase activity compared with soil and Van Hove media.

2- *Azolla pinnata* can be used as water purifier, it can absorb the heavy metal zinc and cobalt from polluted water and accumulate them in its frond.

3- *Azolla* can be totally substitute of a mineral nitrogen fertilizer in wheat production, which lead to reduce environmental pollution caused by continuous application of chemical fertilizer, reduce agricultural costs of wheat cultivation and increase it's grain yield.

Finally we can concluded using *Azolla* in different ecosystems