

CHAPTER I

INTRODUCTION

I N T R O D U C T I O N

Rice being the staple food for more than half of the world's population, its intensive cultivation is a major issue in agriculture. In order to increase yield, high yielding varieties are experimentally selected at various agricultural institutes. Growing more rice requires nitrogen fertilizers in sufficient quantities at a reasonable cheaper rate. The cost of chemical nitrogen-fertilizers has increased tremendously, putting them beyond the reach of a poor farmer. It has therefore become necessary to look for an alternative renewable source of nitrogen to meet at least a part of the requirement of crop production.

Atmospheric nitrogen, of which 78% is in the free state in the atmospheric air, is relatively an inert dinitrogen form. This cannot be directly used by plants to satisfy their nitrogen requirements. In this respect, the role of nitrogen fixing photosynthetic biofertilizer is very significant. The conversion of atmospheric nitrogen into organic compounds by bacteria or by blue green algae is called 'nitrogen fixation' and this process requires expenditure of certain amount of energy. Solar energy is the ultimate source for photosynthesis to produce adenosine triphosphate and a reductant utilized in the nitrogen fixation mechanism.

Prokaryotes probably evolved photosynthetic activity as a consequence of exhaustion of organic substrates, and nitrogen fixing activity as a result of exhaustion of ammonia in the precambrian ocean (Haselkorn, 1978). The genetic information (nif gene) for nitrogen fixing ability is found in prokaryotes. Moreover, nitrogen fixing process is dependent on the nitrogenase enzyme, energy supply, anaerobic conditions and a strong reductant (Fogg et al. 1973). Blue green algae viz. Anabaena, Tolypothrix, Nostoc, Aulosira etc. are known for their nitrogen fixing ability. In some of the nitrogen fixing blue green algae, specialised cells known as heterocysts are differentiated. Heterocysts are thick walled cells and mainly responsible for nitrogen fixation (Stewart, 1973; Haselkorn, 1978). Heterocysts possess a complex controlled lamellar system which protect the nitrogenase enzyme from oxygen (Oppenheim and Marcus, 1970). They carry out photophosphorylation which supplies the necessary energy for nitrogen fixation (Scott and Fay, 1972). The frequency of heterocysts in Azolla-Anabaena complex would therefore determine the quantity of nitrogen fixed. Venkatraman (1972) and Singh et al. (1981) have used blue green algae as biofertilizers and they obtained increase in the yield of rice. However, practical applications of blue green algae in rice fields, face certain difficulties because of

their slow rate of multiplication, quite low quantities of biomass production and their inability to compete with other aquatic flora (Sree Rangaswami, 1980).

Blue green algae fix nitrogen more efficiently when present in symbiotic association in leaves of certain plants such as Azolla. Both Azolla and symbiont Anabaena, harvest solar energy via photosynthesis. The total nitrogen requirement of Azolla could be met by the nitrogen fixed by the symbiont. Therefore, the water fern Azolla is considered as an ideal candidate for nitrogen fixation. . . Gallon (1980) has stated that there remains a need for basic research concerned with the process of nitrogen fixation by the photosynthetic organisms.

Azolla has been used in China and Vietnam for centuries in rice cultivation. Liu Zhang-zhu (1979b) reported that the book Qi Min Yao Shu written in 540 A.D. by Jia Si Xue described the use of Azolla in rice cultivation. Dao and Tran (1979) reported that Beo Giong Azolla and Wild Azolla were the same. Azolla pinnata was the only species native to China. Chinese researchers have been studying Azolla since the early 1950 but many of their achievements were unknown outside China (Lumpkin, 1985). According to Lumpkin, (1985) Chinese are making vigorous efforts to isolate and culture Anabaena symbiont and make intra and inter-specific

crosses and their efforts should not be overlooked. China and Vietnam had a long history of using Azolla in rice production. Azolla plants have been described as miniature nitrogen "fertilizer factories" by Chinese and Vietnamese (Lumpkin and Flucknett, 1982). In India, application studies on Azolla have been conducted at Coimbatore Agricultural University and at Central Rice Research Institute, Cuttack.

Azolla is a free-floating, heterosporous aquatic fern. It belongs to the order Salviniales and is a sole representative of monotypic family - Azollaceae. The sporophyte consists of multibranched rhizome bearing leaves and adventitious roots. Each of the leaf is a bilobed organ and in its dorsal lobe a symbiont, Anabaena azollae Strasburger occurs. It multiplies by vegetative propagation besides through sexual means. It produces two types of sporocarps, develops endosporic gametophytes, and fertilizes gametes resulting in the production of sporophytes.

The review of the literature on Azolla indicates that two distinct aspects have been researched. One aspect deals with basic research, and the other with its applications to crop as a nitrogen fertilizer. Anatomical details (Konar and Kapoor, 1972, 1974), ultrastructure (Duckett et al., 1975) of Azolla and structural inter-relationship of Azolla-Anabaena

complex (Calvert and Peters, 1981, 1982) have been investigated. Reconstruction of serial sections of leaves of various age as defined by their position on the stem axis have provided new insight into the development and organisation of the leaf cavity of Azolla (Hill, 1977). But hardly any anatomical studies have been done on the Indian species of Azolla pinnata correlating with its physiology. While Anabaena is within the leaf, this endophyte within the cavity is extracellular and the actual degree of relationship of the endophyte in the individual species is speculative and constitutes an area of current research (Peters, 1984). Moreover, Azolla is amazing in that it is the only symbiont in which the prokaryotic partner is carried through the sexual cycle of the eucaryote (Peters, 1984).

The basic studies involve increased biomass production of Azolla with simultaneous increase in its capacity for nitrogen fixation. The nutritional requirements of Azolla and its nitrogen fixing symbiont, Anabaena azollae for growth and nitrogen fixation are rather complex and strongly related to the physiology of each of the components of the association (Becking, 1979). The amount of nitrogen fixed by Azolla naturally would depend upon the nutrient status of the culture medium.

The importance of macronutrient elements including phosphorous (P) potassium (K) Calcium (Ca) and Magnesium (Mg) in supporting the growth of Azolla has been recognized. However, quantitative determinations of the nitrogen fixing activity in this symbiosis are rather limited, and information concerning the levels of mineral nutrients required for sufficient display of nitrogen fixation in the symbiosis is almost absent (Yatazawa et al. 1980). Subudhi and Singh (1979^a) have reported that 2/5th dilution of Hoagland's medium as a suitable medium for Azolla culture.

Experimental work reported on A. pinnata (Philippines) showed that growth and nitrogen fixation in mineral deficiencies decreased in the order -K, -P, -Ca and -Mg (Aziz and Watanabe, 1983) while others reported that nitrogen content was lowest in treatments -P, -K, -Ca and -Mg wherein nitrogenase activity was also reduced (Malavolta et al. 1981). The need of phosphorous and magnesium for phosphorylation reactions which provide energy for the endergonic reaction of nitrogen activation explain the reduction in activity found in the corresponding treatments. Phosphorus represents a major limiting factor for Azolla species and strains vary a great deal in their phosphorus requirement (Subudhi and Watanabe, 1981). However, variations have been observed in the degree of reduction in growth of plants cultured in solutions deficient

in macronutrients (Yatazawa et al. 1980). Watanabe et al. (1977), Peters and Mayne (1974a) and Moore (1969) have worked on the nutrient requirements of Azolla but the nutrient requirements of the Indian species Azolla pinnata R. Br. need to be investigated as, this information would help in increasing the yield of Azolla. In addition, efforts should be made in India to screen large number of clones of Azolla, under laboratory conditions for selecting the promising species (Mohan Ram, 1978). Since the amount of nitrogen fixed by Azolla depends on the nutrient status of the culture medium, the mineral nutrition studies would help to formulate/change the mineral composition of known culture media which would support optimal biomass production of Azolla. Growing of Azolla under precisely controlled conditions of environment, as in the case of its axenic cultures would provide unlimited opportunities to study the problems of its organization and physiological processes. However the previous work on Azolla mineral nutrition has not been conducted in axenic cultures.

Regarding the micronutrition of Azolla, molybdenum and cobalt are necessary for nitrogen fixation (Singh et al. 1984). Johnson et al. (1966) showed that cobalt at $0.01 \mu\text{g/l}$ supplied to A. filiculoides cultures growing in nitrogen free medium gave about five times more biomass than in cultures grown in the medium without cobalt. The availability

of minerals to Azolla depends upon the pH of the medium/solution (Nickell, 1958; Ashton and Walmsley, 1976). Olsen (1972) found the maximum dry matter production of Azolla and nitrogen fixation at 6 to 7 pH when iron was present as ferrous ion complex and calcium content (10-20 mg/l) were relatively low.

Since Azolla fixes atmospheric nitrogen through its symbiont Anabaena, presence of nitrogen source in water/culture medium affects its nitrogen fixation. In presence of ammonium sulphate or nitrate in the culture medium, the nitrogen fixation by Azolla gets reduced proportionately (Thomas and David, 1972; Watanabe et al. 1981). Hence, the effect of presence of nitrogen in culture medium of A. pinnata needs to be elucidated.

Although much attention has been paid in recent years to the effects of the gibberellic acid (GA_3) on flowering plants, relatively little importance has been paid to understand the effects of phytohormones on lower plants particularly the pteridophytes (Dusek and Bonde, 1965). In A. mexicana, growth and nitrogen fixation increased by application of gibberellins, (Singh et al. 1984).

The chemical composition of Azolla shows that it is also rich in phosphorus, calcium, potassium, magnesium, iron

besides nitrogen. These chemical constituents showed variations in their quantities according to the availability of the minerals to Azolla during its growth and development (Malavolta et al. 1981).

The second major approach to research on Azolla was concerned with its application to rice crop as a nitrogen fertilizer. Azolla and its symbiont Anabaena both partners harvest solar energy via photosynthesis and the total nitrogen requirement of Azolla gets fulfilled by the assimilation of nitrogen fixed by Anabaena. The relationship between photosynthesis and nitrogen fixation showed that the reductant required for nitrogen fixation was accumulated during light period and that the cyclic photophosphorylation provides a significant fraction of the ATP required for nitrogenase activity (Peters et al. 1980). Azolla no more remains as a botanical curiosity but turns out to be an agronomically useful nitrogen source. Biological nitrogen fixation through Azolla Anabaena complex is considered to be a potential biological system (Kannaiyan, 1979). The opportunity for a concerted approach towards understanding the whole biology of a single species only occurs when commercial interest in the application of this understanding provides the incentive and resources (Dyer, 1985).

Application of Azolla to rice crop as nitrogen fertilizers improved the growth of rice plants and thereby the yield of straw and grain also was increased (Telley et al. 1977). The various factors responsible to bring desired yield in rice grain depends upon the species of Azolla, the quantities used, and methods of its application either by incorporating it in soil or by growing it as a dual crop along with rice.

Singh (1979a) reported that the incorporation of Azolla in soil not only improved the growth of rice plants it also increased the number of tillers, grain and straw yield. Earlier Watanabe et al. (1977) had reported 13% increase in the grain yield when Azolla was incorporated in soil. In the same year Singh (1977), had reported that Azolla incorporated along with 30 kg. or 50 kg. of N/ha as ammonium sulphate caused as much yield as obtained after the applications of 60 or 80 kg/ha of chemical nitrogen fertilizers respectively. More recently Satapathy and Singh (1985) observed significant increase in yield attributing characters such as tillers, panicle numbers and nitrogen content of shoots and roots, when Azolla nitrogen was available to rice crop.

Dual cropping of Azolla along with rice has increased yield from 14 to 40 percent (Moore, 1969). In Azolla filiculoides, dual cropping gave 25% higher yield when compared

with the control (Rains and Talley, 1979). Kannaiyan (1986) used various quantities of Azolla inoculum and found its effect on the yield. Incorporation of Azolla was of greater benefit to rice yield than when it was not incorporated.

Under flooded soil conditions, rate of mineralisation and amount of nutrients released from decomposition of biomass Azolla are important indices of availability of mineral nutrients from Azolla. Understanding these indices is important not only for improving techniques of Azolla propagation and its incorporation, but also for controlling the balance of soil nutrients.

The study of Azolla as a biofertilizer and its application for the improvement of rice crop is very relevant to the Indian situation. Despite several reports of successful application of Azolla to rice crop, its use among Indian farmers is not gaining the required momentum. In order to popularise its application in rice growing areas in India, large quantities of Azolla of desired high nitrogen content should be made easily available to the farmers at a cheaper rate or free of cost all round the year. Hence, studies dealing with maximum biomass production of Azolla pinnata R. Br. need to be conducted.

Another major problem that has been observed in certain areas in India is that many agricultural ecosystems

are increasingly becoming salt affected thus rendering them inhospitable for excellent crop production (Roychoudhary et al. 1985). Very little research work concerning the effects of salinity or osmotic stress on Azolla has been done (Zimmerman, 1985). According to Sukumar and Kannaiyan (1987) the adverse effect of sodium chloride on chlorophyll content was much pronounced in A. pinnata than A. filiculoides. There are hardly any reports on effects of salinity on biomass production of A. pinnata. Benefit of A. pinnata as biofertilizer depends upon its tolerance to the salinity of soil, in which rice is grown. Besides no work has been conducted on the ammonia assimilating enzymes under salinity conditions. Holst and Yopp (1979) reported that in A. mexicana, the effects of salts were more osmotic than ionic. Salt-resistant ecotypes of Azolla species might be existing or may be genetically engineered in the near future. By studying the pigment profile of the endophyte Anabaena of salt treated Azolla, large number of Azolla strains could be screened for their salt tolerance at a faster rate (Rajarathinam and Padhya, 1987 a).

Moreover, there is a report that the physiology of Azolla can be so modified under special laboratory conditions, that hydrogen gas can be generated from water (Mohan Ram, 1978). Hydrogen being a high-energy fuel, the implications of this

discovery are far reaching in view of the sharply declining reserves of petroleum. Newton (1976) also reported the photoreproduction of molecular hydrogen by a plant - algal symbiont system - the Azolla. This is the most challenging achievement about Azolla-Anabaena complex and how far it could be practically feasible depends upon the scientists efforts.

The aspects discussed above form the background for the present work. The main objectives of this work include to elucidate nutritional/hormonal requirements of A. pinnata R. Br. under precisely controlled conditions of environment for its optimal biomass production with simultaneously improved nitrogen fixation capacity. The anatomical details about the in vitro produced A. pinnata and the effects of sodium chloride induced salinity on this complex form the other objectives. Under in vivo conditions the rate of ammonification of A. pinnata as well as its applications as a nitrogen biofertilizer have been evaluated.

The experimental results obtained are incorporated in Chapters III and IV under the following broad heads :-

Chapter III :

In vitro experimental studies on Azolla pinnata R.Br.

a) Nutritional

- b) Hormonal
- c) Anatomical
- d) Salinity

Chapter IV :

In vivo experimental studies on A. pinnata R. Br.

- a) Biomass production
- b) Mineralisation of Azolla nitrogen
- c) Application of Azolla to rice (Paddy)
variety IR 28.