CHAPTER V

GENERAL DISCUSSION

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This research study was developed in two main directions. One dealt with the basic research to evolve a standard method for optimal biomass production under *in vitro* systems of an Indian species of *Azolla pinnata* R.Br., and the other with the applied aspects of *A. pinnata* under *in vivo* systems. This chapter contains a discussion on the results of the different experimental work earlier described under various sections in Chapter III and IV.

Nutritional studies on *A. pinnata* under *in vitro* conditions

Azolla plants being small in size, aquatic in habit with rapid rate of multiplication were ideally suitable as an experimental material for investigating the problems of its organization and physiological processes under *in vitro* systems. The first step of nutritional studies was to develop axenic cultures of *A. pinnata* using a proper sterilizing agent. In the present study, *Azolla* plants were sterilized with hydrogen perioxide and these sterilized plants were inoculated in nitrogen free Watanabe (1977) medium (Expt. No. 1). Sterilization of aquatic plants such as *Azolla* was a rather difficult task, because higher concentrations of sterilizing agents tend to kill the

plants and lower concentrations of the agents appeared to be ineffective in disinfecting Azolla plants. Nickell (1958) had explored that cultures of Azolla after the use of alcohol, sodium hypochlorite and mercuric chloride, as sterilizing agents failed to raise axenic cultures. After two years (1961) he tried penicillin as a sterilizing agent, and established axenic cultures of Azolla but these were without its symbiont Anabaena azollae, therefore its value as a biofertilizer was negligible. By adopting this procedure large number of stock cultures of A. pinnata were kept in continuous culture in culture room. This is the first report on the use of hydrogen peroxide as a successful sterilizing agent, for the establishment of axenic cultures of Azolla.

Another important factor, investigated was the effect of renewal of the culture medium at regular interval for a experimental period of three weeks. Subudhi and Watanabe (1981) obtained higher biomass production in A. pinnata, Bangkok, variety when its culture medium was renewed after every three days. More recently, Salisbury and Ross (1986) opined the composition of the medium changes during the culture period because some of the minerals are more rapidly absorbed than other by the plant.

In the present study, the medium was renewed every week for a period of three weeks. The renewal of the medium was considered to be essential to provide suitable environment to the plants for their growth and multiplication. The renewal of the medium maintained the pH above 5 but below 6, when medium was not renewed, continuous growth caused change in pH, which affected the growth adversely. The exaushtion of nutrients might also be another important factor. It was observed that the biomass production of Azolla was 7-fold in terms of fresh weight after weekly renewal of medium as against 3.7-fold when the medium was not renewed (Expt. No. 2). It was well known that during renewal of the culture medium, aeration of the cultures occurred, which might be responsible for increase in the rate of growth. At the end of three weeks experimental period, the entire surface of the medium was covered by Azolla plants, further increase in the experimental period resulted in over crowding of plants. Hence, in the stock axonic cultures of A. pinnata, weekly renewal of culture medium was carried out for a period of three weeks.

The selection of a suitable medium was based on the maximum biomass production of A. pinnata (Expt. No. 3). Of three known nitrogen free culture media viz. Johnson's

(1966), Peters and Mayne (1974 a) and Watanabe (1977), Azolla recorded highest production of biomass in terms of fresh and dry weights, when grown in nitrogen free Watanabe medium. Moreover, there was no exogenous carbohydrate source incorporated in this culture medium. Azolla fulfilled its energy requirement by synthesizing carbohydrate using solar energy. Due to this aspect, the cost of production of Azolla biomass could be minimised, since quantities of Azotobactor and Rhizobium are being used as the biofertilizers which add to the cost of production. It is heartening to report that Azolla could be used as biofertilizer which would proved to be economical for Indian farmers. The present experiment (Expt. No. 3) established clearly that mineral constituents of Watanabe medium supported growth and multiplication of A. pinnata. Hence, quantity of each mineral element, as present in this medium was considered to be as a standard dose. All further experimental work on Azolla pinnata was done using this medium.

The major factor that effects the growth and nitrogen fixation of Azolla is the pH of its culture medium. Epstein (1972) had shown that pH of the culture medium plays an important role in the mineral absorption by plants. According to Salisbury and Ross (1986), the pH of culture

medium strongly influences the uptake of the phosphate because it influences the ionic charge. Plants utilised monovalent forms of minerals more readily than divalent and trivalent forms.

In the present studies on A. pinnata, out of the various pH range tested in the nitrogen free medium, it was observed that plants grown in medium at pH 5.5 and at 6.5 were dark green, with well developed root system. Besides, at this pH of media, the biomass produced was significantly higher in terms, of fresh and dry weights. The chlorophyll contents and nitrogen contents were at their optimal levels (Expt. No. 4). The other growth parameters examined in Azolla, viz. the heterocyst frequency and acetylene reduction activity, were also at their optimal levels, at pH 5.5 and 6.5 range of the media. The heterocyst frequency was 30% in the Anabena azollae isolated from Azolla cultured in medium at pH 5.5 and 6.5. At higher pH 7.5 of the medium these growth parameters showed reduction. Fiori and Ruschel (1981) observed that in A. filiculoides the nitrogen content was higher at pH 5.5 while nitrogenase activity was higher at pH 6.0 and 6.5. In other species of Azolla, namely A. mexicana, Holst and Yopp (1979) found 6.5 as the optimal pH of the culture medium which supported maximum biomass production. In 1979,

Becking working with A. pinnata observed that the pH 5.5 to 6.5 of irrigation water in which Azolla was grown, supported optimal biomass production. Watanabe et al. (1977) pointed out that at higher pH levels of the culture medium, iron was not available to Azolla plants, which might have been one of reasons of decreases observed in biomass production. The authors suggested that at higher pH levels of the culture medium, ferric ion precipitates as the insoluble ferric hydroxide which might not be absorbed by Azolla plants (Epstein, 1972; Hewitt and Smith, 1974). In the present study, at lower pH 4.5 level of the medium, biomass production of Azolla showed a decline. This was attributed to the fact that phosphorus in the medium at low pH precipitated as calcium and magnesium phosphates and became less available to the plants (Lumpkin and Plucknett, 1980). Moreover a pH range of 5 to 8 of culture medium kept in open container supported Azolla biomass production, optimal production being at pH 6 (Subudhi and Singh, 1979). The discrepencies in the results might be due to the differences in Azolla strains and the variations in their cultural conditions.

Azolla growth is frequently limited by nutrient levels in aquatic systems (Kannaiyan et al. 1981 a). Among the various nutrients, phosphorus, potassium, calcium and

magnesium are important as macronutrients (Peters, 1977) while iron, molybednum and cobalt as micronutrients have been shown to be essential for the growth and nitrogen fixation of Azolla (Moore, 1969).

Plants grown in solutions difficient in macro and microelements exhibited symptoms of nutrition deficiencies. It was seen the deficiency symptoms depends upon the severity of the deficiency as well as on the particular species or strain of plant and on many other enviromental factors (Episten, 1972). In deficiency, the overall growth and development of plants are affected.

In the present studies (Expt. No. 5) concentrations of individual ions in the nitrogen free Watanabe medium were altered one by one to explore the optimal level of ions that would support maximum biomass production of A. pinnata under controlled environmental conditions. Care was taken to eliminate the carry-over effects of the ions.

As early as in 1963, Nason and Mc Elroy, reported that the potassium in highest concentration was found in the meristematic regions of the plant, an observation that supported with the finding of Webster and Varner (1954). In addition, to its role as an activator in protein metebolism, potassium also acts as an activator for several

enzymes involved in carbohydrate metabolism and therefore is needed in optimal level in culturing of A. pinnata plants.

In the present investigation, deficiency studies of potassium in A. pinnata revealed that there was negligible growth and multiplication in absence or at 10 ppm level of K. Leaves showed chlorosis and roots were poorly developed. The other growth parameters viz. chlorophyll, protein, contents showed decline by 29% and 26% respectively. Similarly the heterocysts frequency of the symbiont was reduced bringing corresponding decrease in acetylene reduction activity. The optimal biomass production in terms of fresh and dry weights of Azolla was recorded at 40 ppm of K level in the culture medium. Nitrogen content was though not significantly increased at 40 ppm of K level in the culture medium was higher than the rest of the levels. Heterocyst frequency and nitrogenase activity showed that 40 and 50 ppm of K levels supported significantly superior nitrogenase activities. Of these two levels, 40 ppm of K level was superior in supporting biomass production of Azolla and nitrogenase activity, when compared with these parameters at 50 ppm of K level. All these results were obtained from in vitro grown A. pinnata under constant temperature and photpperiod.

Magnesium, being a constituent of the chlorophyll molecule its deficiency adversely affects the photosynthetic mechanism of Azolla in culture. Magnesium besides being a constituent of chlorophyll molecule, is also involved in carbohydrate and protein synthesis as an activator in some of the enzyme systems (Devlin and Withem, 1986). Magnesium is a co-factor of many enzymes which act on phosphorylated substrates thereby it is of paramount importance in energy transport (Epstein, 1972).

Azolla pinnata cultured in medium lacking magnesium caused chlorosis on the leaves, initially noticed upon the older and later on the younger leaves as well. Roots were detached from the plants. Biomass production of Azolla was reduced by 67% and chlorophyll content was reduced by 32% when compared with Azolla grown in standard dose of Mg (40 ppm). Increasing the levels of Mg in the culture medium upto 40 ppm showed linear increase in biomass production of Azolla and an improvement in all other parameters studied. Heterocysts frequency was at its optimal level thereby indicating maximum acetylene reduction activity.

The functions of calcium in plant growth appear to be multiferious. It is a major constituent of (calcium pectate) the middle lamella of the cell wall and thus is

responsible for mechanical strength to the tissues (Ito and Fijiwara, 1967). Calcium deficiency leads to the disorganisation of cells and tissues, suggesting that it is needed for maintaining the cell membranes in a functional state (Hewitt and Smith, 1974). Calcium is an activator for the enzymes adenosine triphosphate, adenyl and kinase etc. (Mazia, 1954). Norris and Jensen (1957) found that calcium was required for the growth and nitrogen fixation of Azotobacter.

In complete absence of calcium in the culture medium, A. pinnata plants were reduced in size and roots remained small in size. The biomass production of Azolla was reduced by 74% when compared with plants grown in standard dose of calcium (40 ppm). Chlorophyll, protein and nitrogen contents were reduced by 48%, 27% and 26% respectively. Heterocyst frequency was reduced by 32% while ARA recorded 74% reduction. At 40 ppm of calcium in the medium optimal biomass production of Azolla along with all the other growth parameters was observed. A positive correlation existed between biomass production of Azolla and its nitrogenase activity ($r = 0.99$) and similarly between the dry matter of Azolla and nitrogen content ($r = 0.988$).

Phosphorus is found in plants as a constituent of nucleic acids, phospholipids and adenosine triphosphate.

It being a component of adenosine triphosphate, it forms a part and parcel of the universal energy currency of all the living cells (Epstein, 1972).

The symptoms developed due to phosphorus deficiency in Azolla were, growth arrest, decreased frond size, fragility in plants, and marked browning of roots. In absence of phosphorus there was 80% reduction in growth of Azolla as compared to plants grown in standard dose of phosphorus (20 ppm). The increase in phosphorus level from 20 to 30 ppm. supported maximum biomass production of Azolla besides supporting its nitrogenase activity. Hence for optimal biomass production of A. pinnata, phosphrus level in the culture medium needs to be raised to 30 ppm.

Iron is constituent of the enzyme nitrogenase (Gallon, 1980). Moreover, iron is a constituent of cytochromes responsible for the electron transport system (Epstein, 1972, Gauch, 1972). Iron has been identified as a compound of various flavoproteins active in biological oxidation.

Fragmentation, chlorosis on leaves, and browning of roots were some of the visible symptoms noticed in Azolla plants in response to absence of iron in culture medium. Biomass production of Azolla exhibited 70%

reduction. But at 2 ppm of Fe, in culture medium improved the biomass production of Azolla. In addition, the chlorophyll content and nitrogenase activity were also at their maximum at 2 ppm of iron level.

The experimental studies conducted on A. pinnata under in vitro conditions showed that Azolla required all mineral elements for its growth and biomass production. The levels of Mg, Ca, K and Fe as present in the Watanabe medium were found to be optimal levels for maximum biomass production of the Indian species of Azolla. However, phosphorus level has to be raised to 30 ppm to achieve maximum biomass production of this Indian species.

Results with various species of Azolla such as A. pinnata, A. mexicana A. caroliniana and A. filiculoides exhibited substantial differences in the phosphorus requirements (Subudhi and Watanabe, 1981). Phosphorus has been shown to be essential for the rapid multiplication of Azolla and nitrogen fixation (Moore, 1969; Tran and Dao, 1975). Singh and Srivastava (1984) observed that in A. pinnata, calcium and phosphorus deficiencies reduced growth but at the same time increased soluble sugar content in the plants. In A. imbricata plants disintegrated when they were deprived of magnesium. Yatazawa et al. (1980) indicated

that deficiencies of mineral elements resulted in reduction of various growth parameters in Azolla.

The variations in their results might be attributed to the different strains of Azolla used besides the unidentified experimental conditions. Watanabe and Berja (1983) found A. pinnata possessed tolerance to high temperature.

Singh (1979a) found that absence of calcium in the culture medium, Azolla lacked its symbiont. Aziz and Watanabe (1983) found that mineral deficiencies in the culture medium for Azolla decreased its nitrogen fixing ability. In the Medicago sativa - Rhizobium milioti - symbiotic relationship, a calcium deficient condition decreased the growth and nitrogenase activity (Miller and Sirois, 1983). The study clearly suggested that A. pinnata required requisite quantities of standard dose of macro-elements for its biomass production and nitrogenase activity. Results of the various species of Azolla studied by others also are in agreement with the studies of the present work.

Watanabe medium lacks cobalt, and it was desirable to find out effects of addition of cobalt in the medium on the biomass production of Azolla. Cobalt when supplied

in trace quantities had been shown in legumes to increase nitrogen fixation (Powrie, 1954). But its effect on nitrogen fixation in blue-green algae alone or in its symbiotic association has not been investigated. It was found in the present studies that addition of 0.1 ppm of cobalt, to the culture medium, improved the biomass production of A. pinnata by 24% (Expt. No. 6). The nitrogenase activity also showed improvement over the nitrogenase activity exhibited by Azolla grown in the standard medium without cobalt. Johnson et al. (1966) found that cobalt was required for the growth of Azolla filiculoides and without cobalt, growth was restricted and a severe chlorosis typical of nitrogen deficiency developed. The necessity of cobalt for growth of certain blue-green algae (Ford, 1953), for symbiotic nitrogen fixation by legumes (Ahmed and Evans, 1959) and for certain non-legumes (Bond & Hewitt, 1962) had been reported. However, Johnson et al. (1966) observed the increase in biomass of Azolla without the addition of cobalt to the medium which the authors attributed to cobalt initially present in the plant tissue or traces of the metal present as an impurity in the culture medium. But when they added cobalt to the medium, they observed a marked increase in the number of algal cells per gram fresh weight of Azolla fronds. But

in the present studies, since double glass distilled water was being used, there was no likelyhood of cobalt remaining as impurity, therefore increase in biomass production of Azolla was due to addition of cobalt perse.

Wahal et al. (1973) working with blue green algae Anabaena ambigua showed that application of 0.5 µg/l ascorbic acid increased 3-fold heterocyst frequency and thereby nitrogenase activity was also increased. They suggested, that ascorbic acid might be playing a role in maintaining the highly reduced conditions inside the heterocyst, needed for effective functioning of nitrogenase. Therefore, it was interesting to study the effect of ascorbic acid on the Anabaena which is symbiotic in A. pinnata.

A. pinnata under investigation showed that incorporation of ascorbic acid at 10 ppm in the culture medium, increased its biomass production by 14%, heterocysts frequency by 10% and nitrogenase activity by 9%, when compared with Azolla grown without ascorbic acid (Expt. No. 7). A positive correlation existed between biomass production of Azolla and its nitrogenase activity (r=0.973).

It has been reported that nitrogen supplied in the form of ammonium salts in particular, suppressed both

heterocyst frequency and nitrogenase activity (Peters et al. 1979; Peters and Ito, 1984). Addition of combined nitrogen in the culture medium of blue green algae, affected considerably the nitrogenase activity because its presence caused, inhibition in the synthesis of nitrogenase enzyme or it inhibited the nitrogenase or it created breakdown in existing nitrogenase (Fogg et al. 1973). Higher concentrations of combined nitrogen, present in the culture medium inhibited heterocyst differentiation and thereby reduced nitrogenase activity (Stewart et al. 1968; Ogawa and Carr, 1969).

The effects of exogenous supplied nitrogen, on the growth of A. pinnata and its nitrogenase activity were investigated. The nitrogen (40 ppm) was added to the Watanabe medium, in which A. pinnata was grown, there was no reduction in biomass production, chlorophyll content and protein contents (Expt. No. 8). When nitrogen was supplied as ammonium sulphate there was reduction in the heterocyst frequency by 21% causing reduction in nitrogenase activity by 59%. Similarly when ammonium nitrate was the nitrogen source added to the culture medium, there was reduction in heterocyst frequency and nitrogenase activity without reduction in biomass production of Azolla and its composition. The percentage reduction in the

frequency of heterocyst and in the nitrogenase activity were more when ammonium salts, than when calcium and potassium nitrates were used as the sources of nitrogen.

Nitrogen free Watanabe medium supported optimal biomass production of A. pinnata, clearly showed that the nitrogen fixed by its symbiont Anabaena azollae fulfilled the total nitrogen requirement of the fern (Moore, 1969). Thus it seems that the decrease in nitrogen fixation by the algae is compensated by nitrate utilisation, maintaining a constant nitrogen content and biomass production of A. pinnata (Peters and Mayne 1974b; Peters et al. 1980). Besides that nitrogenase activity of the symbiont Anabaena is protected by the fern from combined nitrogen added to the medium (Peters and Mayne, 1974b). Peters et al. (1977) reported, that significant nitrogenase activity is retained by the symbiont when the association is grown in combined nitrogen. This supports the results obtained in A. pinnata, since it was noticed that in the presence of ammonium or nitrate salts certain amount of nitrogenase activity occurred.

A. caroliniana, when grown in nitrogen containing medium, the growth rate and chlorophyll content remained quite constant with increasing nitrate concentrations

upto 25 mM in the culture medium (Peters et al. 1981). Since individual, phosphorus, as well as cobalt had promoted biomass production of Azolla (Expts. 5 and 7) it was worth while to modify the medium to study the combined phosphorus and cobalt effects on the biomass production of A. pinnata (Expt. No. 9). A. pinnata cultured in modified Watanabe medium containing 30 ppm P and 0.1 ppm Co, supported a 2-fold increase in biomass production over Azolla plants grown in standard Watanabe medium. There was 30% increase in the biomass produced over the control. Moreover, the heterocyst frequency showed 17% increase with 9% increase in acetylene reduction activity in A. pinnata cultured in modified medium. Statistical analysis of the data also confirmed this result.

Azolla pinnata grown on semi-solid medium (Expt. No. 10) showed that it could survive for a period of three to four months without losing its potential for further multiplication. However, the biomass production was 2.5-fold in terms of fresh weight, while in liquid medium it was increased by 3.7-fold as seen from the result of experiment No. 2. Still on solid medium, renewal of the medium was not done and the biomass production was compared with that of in the liquid medium where a weekly renewal was not carried out. Culturing of A. pinnata on

a semi-solid medium provided the techniques that preserved the germplasm of A. pinnata (Rajarathinam and Padhya, 1987b). Since, Azolla was cultured in petri-dishes on semi-solid medium it could be easily transported. If proper measures to preserve the pteridophyte flora are not taken up by setting up germ-plasm banks and by other means, then a time will come when ferns would be things of past beauty (Gomez-P, 1985).

In the present experimental studies conducted under in vitro systems on A. pinnata, the Indian species confirmed that it required all the usual macroelements as present in nitrogen free Watanabe medium (Padhya, 1987). As regards its requirement for phosphorus it was slightly higher (30 ppm) than what is present in the original medium.

Hormonal studies

Phytohormones are regulators produced by plants, which in low concentrations regulate plant physiological processes in general. Hormones usually move within the plant from a site of production to a site of action. Kinetin promotes cell division besides cell enlargement. Mothes and Engelbrecht (1961) demonstrated the Kinetin - induced transport of soluble nitrogen from intact leaves of Nicotina rustica to other leaves on the same plant.

Witham and Millar (1965) have speculated that equivalent levels of active cytokinins in physiologically active leaves and stems, regulate nutrient flow so that the nutrients are drawn to certain areas while the plant is in the vegetative state.

Use of phyto hormones for faster growth and multiplication in A. pinnata was studied under in vitro systems. The various functions of growth regulators prompted us to investigate, whether any one of the hormones would improve the rate of biomass production of Azolla pinnata as well as its nitrogenase activity.

The application of kinetin at various levels to A. pinnata brought about slight increase in biomass production (Expt. No. 11). Similarly application of indole-3-acetic acid (IAA) showed negligible increase in biomass production of A. pinnata. However, Nickell (1961) found at 0.1 ppm of IAA level was stimulating for growth of A. caroliniana and in A. mexicana, it caused a slight reduction in fragmentation. Dusek and Bonde (1965) treated plants with IAA levels and found, no effect on biomass production in Azolla. Usually in ferns the multiplication of new shoots occurs by lateral bud formation.

The application of 2, 4-D at various concentrations to A. pinnata showed no improvement in biomass production. As a matter of fact higher levels of 2, 4-D were found to be toxic to A. pinnata resulting in death of the plants. Nickell (1961) had observed similar result. In A. caroliniana Venkatraman and Rajalakshmi (1971) have reported that application of 2, 4-D to blue green algae adversely affected their nitrogen fixation.

Allsop (1963) found that in Marsilea, plants grown in 4% glucose medium showed land forms but incorporation of 10 ppm GA_3 caused the transformation of land forms to water forms. In the present study gibberellic acid in low concentrations incorporated in the culture medium increased biomass production of A. pinnata. The increase in biomass production recorded was by 10% and in acetylene reduction activity by 5% when compared with plants grown in standard medium. Singh et al. (1984) have also reported that GA_3 treatment to A. filiculoides and A. pinnata (Vietnam) showed increased biomass production and nitrogenase activity.

These studies showed that the exogenous application of phytohormones such as kinetin, indole-acetic acid and 2, 4- dichorophenoxy-acetic acid were not required for

biomass production of A. pinnata. This might be due to the fact that the endogenous levels were sufficient enough for growth and development of these plants. However, gibberellic acid at low concentrations promoted biomass production of A. pinnata. In general, it may be concluded that addition of phytohormones did not have any dramatic effect on the growth parameters selected in this study.

Anatomical studies

In vitro grown Azolla from stock cultures was used to study the anatomical details of A. pinnata (Expt. No. 12). It was observed that the shoot apex of Azolla was surrounded by Anabaena cells. From the first leaf onwards, Anabaena azollae was found to be localised inside the dorsal leaf of Azolla. The dorsal leaf lobe cavity was lined by epidermal cells which showed the presence of two types of outgrowths in the form of hairs. The number of branched hairs in all dorsal leaf lobes, counted upto 22nd leaf in serial order of development, was found to be only two. These two branched hairs are located in similar positions in every leaf cavity, always on the path of the foliar trace.

The simple unbranched hairs showed gradual increase in number upto 22, from 1st leaf onwards upto 15th leaf.

The number of simple hairs were found to be the same from 15th to 20th leaves.

Multicellular simple or branched hairs develop the structural characteristics of transfer cell but not simultaneously in A. caroliniana (Calvert et al. 1985). The host received and utilizes nitrogen fixed by its endophyte and Anabaena received fixed carobon from Azolla (Kaplan and Peters, 1981; Peters et al. 1985). Peters et al. (1985) found sucrose synthesized by Azolla taken up by Anabaena. The simple hairs are comprised of only two cells, a stalk cell and a terminal cell. Anabaena trichomes present in the leaf consisted of vegetative cells and heterocysts. The frequency of heterocysts counted from isolated Anabaena of the first leaf onwards showed progressive increase upto 30%. Heterocysts being the site of nitrogen fixation (Haselkorn, 1978), the increase frequency reflects the improvement of nitrogen fixing capacity of Azolla (Peters and Mayne, 1974b). Venkatraman (1983) reported that nitrogenase is oxygen sensitive and different types of structural and functional oxygen protective mechaniams are observed among blue green algae of which the most important type is the heterocyst. Peters (1975) reported in A. caroliniana, the heterocyst

frequency was 21% and vegetative cells 62% from all stages of leaf development. Heterocyst frequency increased from zero in the apex to above 30% in mature leaves (Hill, 1975). Kaplan et al. (1986) reported that nitrogenase activity was negligible near the apex. Ito and Watanabe (1985) reported that acetylene reduction activity was more in young actively growing Azolla fronds than the old ones. This might be due to the maximum heterocyst frequency upto 20th leaf and this anatomical studies have thrown light on the physiology of A. pinnata.

Under flourescence microscope in A. pinnata, vegetative cells and heterocysts of Anabaena, showed the presence of chlorophyll and phycocyanin pigments. The same results were observed in other species of Azolla (Ray et al. 1978; Tyagi et al. 1981). But Becking and Donze (1981) found higher chlorophyll contents than phycocyanin contents in Anabaena isolated from A. carboliniana and A. pinnata variety pinnata. Phycocyanin accounts for about 70% of the total phycobiliproteins while phyoerythocyanin and allophycocyanin accounts for about 17% and 13% respectively (Tyagi et al. 1980). Phycobiliproteins of heterocysts of blue green algae are associated with photosystem II (Haselkorn, 1978).

Anatomical studies on A. pinnata cultured in vitro in nitrogen free Watanabe medium showed that the structural details were identical to the Azolla grown in nature. Besides, structural interdependence between the host and the endophyte, Anabaena azollae was observed. This experiment showed that Azolla artificially cultured under the conditions, were not different in their anatomical structure and were as normal as those growing in nature.

Salinitry studies

Many agriculture ecosystems are increasingly affected due to salinity. Roychoudhary et al. (1985) have stated that presently about seven million hectares of potential crop land in India has been salt affected.

Since Azolla is being considered as a nitrogen fertilizer and since the salinity of soil decreased availability of nitrogen to the crop, the effects of salinity were investigated (Expt. No. 13). Azolla plants subjected to low salinity 5 mM and 10 mM showed no change in biomass production as well as acetylene reduction activity. But with the increase in sodium chloride level to 20 mM Azolla plants became pale green, roots remained short and were dark brown. Further increase in sodium chloride level to 40 mM, biomass production of Azolla was decreased by 72%.

Chlorophyll and protein contents also were reduced. Nitrogen content decreased by 31%, acetylene reduction activity by 69% when compared with corresponding values of Azolla plants grown in standard medium without sodium chloride. Thus it was quite evident, that A. pinnata could tolerate salinity induced by 10 mM sodium chloride on its growth and nitrogenase activity when grown under in vitro systems, where the temperature and light intensities were constant (Rajarathinam and Padhya, 1987c).

Singh and Srivastava (1984) found that A. pinnata grown in 1.5 and 2.0 % salt solutions died within four days. Sukumar and Kennaiyan (1987) working on A. filiculoides and A. pinnata, found that sodium chloride adversely affected nitrogenase activity and nitrogen contents but the effects on biomass production were not to the same extent. According to Peters and Mayne (1974b) there is close relationship between photosynthesis and nitrogen fixation. Sodium chloride induced salinity reduced chlorophyll content, thereby adversely affecting the photosynthetic process. This might explain the reduction in nitrogenase activity as a result of less availability of energy. The decrease in protein contents in response to sodium chloride treatment might be due to the stimulation of protein hydrolysis (Sharma and Gupta, 1986).

Very little work has been conducted concerning the effects of osmatic stress or salinity on Azolla. Holst and Yopp (1979) compared the effects of sodium chloride and polyethylene glycol (PEG) 6000 on A. mexicana and found very slight decrease in biomass production at 2 ppt and 4 ppt of sodium chloride treated Azolla plants. According to them, the effect of lowered water potential due to PEG parallels that due to salt, indicating more of an osmotic than ionic effect produced by salts.

In A. caroliniana and A. filiculoides, both exceptionally salt tolerant species, did not tolerate conditions of 5 ppt seawater salts, which technically qualifies as a saline environment (Zimmerman, 1985).

Ammonia assimilating enzymes from sodium chloride treated Azolla pinnta were estimated. At low levels of sodium chloride (5 mM) the glutamine synthetase (GS) was reduced by 6% while glutamate dehydrogenase (GDH) and glutumate synthase (GOGAT) were unaffected. With increase in sodium chloride level to 20 mM, GS activity was reduced by 39%. Further increase in sodium chloride level to 40 mM, GS activity reduced still further by 77% when compared with GS activity shown by Azolla cultured in standard medium without sodium chloride. But the GDH and GOGAT

activities were not affected to that extent due to the presence of sodium chloride levels in A. pinnata culture medium. At 40 mM, of sodium chloride, GS was reduced by 77% while GDH and GOGAT were reduced by 13% and 8% respectively. This clearly indicated that the GS activity of A. pinnata is very sensitive and gets reduced by the presence of sodium chloride in the medium. The characteristically low levels of ammonia assimilating enzymes of the symbiont Anabaena azollae makes the nitrogen fixation process very efficient (Peters et al. 1980).

Ray et al. (1978) found that in the Azolla Anabaena association, both partners possess the capacity for glutamate synthesis through GDH or by GS - GOGAT pathway. The host Azolla contributed about 90% of the total GS and 10% of GDH activities (Peters, 1979). Kannaiyan and Venkataraman (1985) in their field experiments observed that presence of nitrogen source added to the soil markedly suppressed the GDH activity of Azolla but not GOGAT and GS to that extent. GS and nitrogenase interact in blue green algae and the general consequence is that GS is involved directly or indirectly in nitrogenase regulation (Nagatani et al. 1971; Kannaiyan, 1986).

Ionic constituents of A. pinnata grown in culture media containing sodium chloride at various concentrations

showed that it changes according to the levels of sodium chloride present in the medium. At 40 mM of sodium chloride present in the medium, sodium and chloride ions in A. pinnata were increased by 77% and 151% respectively, when compared with the corresponding values of Azolla grown in standard medium. Potassium, calcium and magnesium contents showed reduction by 10%, 34% and 62% respectively. It is postulated that once Azolla is incorporated in the soil, it decomposes releasing potassium in the soil. Hence Liu Chung Chu (1984) thought that Azolla could be an ideal source of potassium to the rice crop besides being the nitrogen source. The growth reduction observed in many of the glycophytes might be due to the passive accumulation of sodium which creates an "ion excess" sphere inside the plant (Greanway and Munns, 1980). In the present studies, Azolla pinnata behaved like other glycohytes in accumulating sodium ions at higher salinity levels. Similar results have been observed in Vigna Sinesis (Imomul Haq and Larher, 1984). In Sorghum plants, high salinity levels have slightly decreased magnesium content (Patel et al. 1975). Imamul Haq and Larher (1983), Yeo and Flowers (1985), found that chloride contents increased in other plants under salinity conditions. These findings support our results with A. pinnata.

The results obtained on A. pinnata grown in sodium chloride incorporated medium clearly indicated that as the levels of sodium chloride increased, the accumulation of sodium and chloride ions increased proportionately. Azolla grows as a weed, could be used as a salinity removal from salinity affected areas, to some extent. Further research in this direction might help to solve the problems of salinity in soil/water to certain extent by using Azolla as an agent.

The measurement of chlorophyll and phycocynanin pigments of the endophyte Anabaena, isolated from salt treated Azolla using flourescence microscope provided a non-destructive rapid method for obtaining information, on the mechanisms of salt action on pigment profile. Chlorophyll and phycocyanin contents of vegetative cells and heterocysts of Anabaena, showed gradual reduction in their quantities as the level of sodium chloride in the culture medium increased. At 40 mM of sodium chloride, chlorophyll contents disappeared from both the cell types while slight phycocyanin content remained in the heterocysts. In free living Nostoc muscorum, phycocyanin contents were reduced due to salinity (Blumwald and Tel-Or, 1982). Ray et al. (1979) reported that light

energy absorbed by phycobilins effectively promoted nitrogenase activity. Allen and Smith (1969), Van Gorkom (1971) observed nitrogen starvation in blue green algae leads to the disappearance of phycobilin pigments, which actually serves as a reserve source of nitrogen. Kaplan et al. (1986) provided quantative information on the pigment contents an unequivocal evidence for the occurrence of phycobiliproteins in both the cell types, vegetative cells and heterocysts of Anabaena in A. caroliniana and A. pinnata.

In conclusion, in vitro studies on sodium chloride induced salinity on A. pinnata, provided ample scope for using this information while putting Azolla to practical use. In addition, the flourescence method using microscope provides a relationship between the level of sodium chloride in the culture medium and the pigment profile, which can be used for screening out the strains of salt resistance of Azollas.

In vivo studies on A. pinnata R. Br.

Biomass production

To conduct experimental work on the application of A. pinnata R. Br. large quantities were required. Hence, A. pinnata was grown under in vivo system in plastic trays

in the modified Watanabe medium, which was proved to be superior for its optimal biomass production under *in vitro* conditions (Chapter III). Growing of *A. pinnata* in plastic trays using modified Watanabe's medium, showed that its biomass could be increased by 12-fold, in terms of fresh weight, whereas only 8-fold increase in biomass production was obtained in Watanabe medium. Plant nutrition is very important for the successful cultivation of *Azolla* and *Azolla* being an aquatic plant, derives its nutrition from the medium in which it grows.

Recently mass propagation of *Azolla* species have gained movementum and Action Programmes at national level have been introduced all over the East (Annoymous, 1985, 1986, 1987). However unless effective procedures are worked out for *Azolla* multiplication locally, no one should attempt to exploit its commercial application (Lumpkin and Plucknett, 1982). The F.A.O. team is at present testing several strains of *Azolla* in a programme aimed at introducing *Azolla* at the farm level (Van-Hove, 1987). Moreover, animal nutrition experts have shown that *A. caroliniana* is the most nutritious species when compared to *A. microphylla* and *A. pinnata* (Annynomus, 1985). In light of its potentialities, National Azolla Action Programmes were directed at generating, refining and

spreading of Azolla technology in answer to real, pressessing needs in Philippine agriculture (Annynomus, 1986). There is a real need in India, to develop a similar National action programme initially mass culturing of Azolla which could form a source of supply.

In the present studies large mass of A. pinnata was obtained by in vivo growing Azolla in modified Watanabe medium.

Mineralisation of Azolla nitrogen

The nitrogen contents accumulated in Azolla becomes available to rice plants only when it starts decomposing in flooded soil.

The comparative analysis of decomposing of A. pinnata at different growth stages showed, that 50% amonification of vegetative Azolla was completed at the end of fourth week, while for sporulating Azolla, it took period of five weeks. By the end of 8th week, about 80% ammonification occurred from Azolla in both the stages of its development (Rajarathinam and Padhya, 1986). Earlier, Watanabe et al. (1977) obtained similar results. However, they found that ammonia released at a faster rate from Azolla mixed in flooded soil in fresh condition than in dry condition. In A. imbricata and A. filiculoides, rates

of ammonification peaked during 2nd to 3rd week period after incorporation of the plants in the flooded rice soil (Shi-ye-Li, 1981). Singh (1979b) showed that rate of ammonification in Azolla at room temperature was faster than that at 24° + 2°C.

Mian and Stewart (1984) reported that Azolla nitrogen starts releasing ammonia after incorporation of Azolla in the flooded soil. Ammonia accumulated into the soil, in the first one month but it gradually disappeared in the following month instead of being retained in the soil. A. pinnata grown in Watanabe medium showed the capecity of releasing its nitrogen contents after one week of incorporation in the flooded soil. Besides, nitrogen released from Azolla when it was in its vegetative stage or sporulating stage of development, was almost the same.

The chemical composition of A. pinnata, showed that its ash contained 3.3% potassium, 1.25% phosphorus, 1.14% magnesium and other elements. When A. pinnata is incorporated in rice fields, besides adding nitrogen to the soil it adds all other minerals present in it thereby it improves the soil texture. Liu Chung Chu (1979) found that different samples of Azolla collected from different

localities during various seasons of the year contained phosphorus and potassium but their quantities differed. A. microphylla contained more of potassium but low iron, manganese, and copper contents (Shi-ye-Li, 1981). The phosphorus content was very low in sporulating Azolla (Lumpkin and Pluck nett, 1982). Even though A. filiculoides and A. microphylla were grown under identical conditions, A. microphylla contained calcium and iron more while A. filiculoides contained more of potassium (Lumpkin and Plucknett, 1982). The dry weight of Azolla has been reported to range from 4.8 to 7.5% (Peters and Calvert, 1982). Liu Chung Chu (1984) reported, that Azolla possessed rather strong capacity for absorbing potassium from water and thus it could be an excellent source of potassium to rice crop.

Application of Azolla as biofertilizer to rice variety IR 28

It has been an established fact that to have more yield of rice, not only high yielding variety could solve the problem, it needs adequate amounts of nitrogen fertilizers. A. pinnata grown in plastic trays was used for evaluating its potential for increasing rice yield.

In the pot experiments conducted on rice variety IR 28, Azolla alone, ammonium sulphate alone, Azolla

combined with ammonium sulphate were incorporated in the soil and a control received no treatment (Expt. No. 17). Experiments conducted during the summer and kharif seasons of the year 1986, clearly showed that at 30 days, height of rice plants was significantly more with Azolla application than that of the control. But ammonium sulphate application alone showed still further improvement in the height of rice plants. When Azolla was combined with ammonium sulphate as a nitrogen fertilizer, the height of rice plants further increased. Azolla inoculation alone had profound effect on the growth of rice roots. Besides that the biomass production of rice and its nitrogen content were equally improved by application of Azolla /ammonium sulphate/ Azolla combined with ammonium sulphate.

At 60 days and 90 days of planting of rice seedling, the results on all the growth parameters such as biomass production of rice plants in terms of fresh and dry weights, nitrogen contents in them besides the shoot-root length, gave indentical information proving A. pinnata supplied as nitrogen fertilizer was superior over the control. But Azolla combined with ammonium sulphate was far better than Azolla alone or ammonium sulphate alone. The use of Azolla and ammonium sulphate increased rice dry matter significantly higher than that of the dry matter produced in rice

plants grown without addition of any fertilizer. Mian and Steward (1985) using ^{15}N tracer study, compared nitrogen supply by Azolla and ammonium sulphate to rice variety IR 8 under flooded conditions and reported that more nitrogen was available to the rice plants from ammonium sulphate than from Azolla. Rice plants over 60 days of planting received 34% ^{15}N applied as Azolla while they received 61% ^{15}N as applied as ammonium sulphate. This indicated that Azolla supplied half the quantity of nitrogen when compared with ammonium sulphate.

In the present experiment, at 60 days, 90 days and at the time of harvest the rice plants showed significantly higher number of tillers and panicles produced in rice plants treated Azolla than without it. The number of productive tillers were significantly increased due to Azolla inoculation. Similar results were reported by Kannaiyan and Rajeshwari (1983). Singh (1979a) conducted pot experiments after incorporation of Azolla in the soil and reported that growth of rice plants, number of tillers, grain and straw yield were more than the control. Moreover Singh (1977), in his previous report indicated that the increase of rice grain yield was directly proportional to the quantity of Azolla incorporated in the paddy soil. Azolla inoculum should be incorporated in the soil after the establishment of rice seedlings in case of dual crop

(Singh, 1979a). Talley et al. (1977) reported 23% and 67% increase in yield of rice by the application of A. filiculoides and A. mexicena, thereby indicating the variation in the availability of nitrogen from two different species. Further research needs to be done on various species of Azolla to find out their competence as nitrogen fertilizers. Thuyet and Tuan (1973) reported that the utilization of Azolla as a biofertilizer for increasing rice yield is a customary part of rice culture in north Vietnam and presently about 900,000 hectare of land received Azolla as nitrogen fertilizer.

Mian and Stewart (1985) presented a data whereby they showed that the dry matter produced in rice variety IR 8 increased by 74%, 105% and 125% due to the increased uptake of nitrogen from Azolla, Anabaena and Nostoc in sixty days of applications, when compared with the control rice plants. They further showed with the use of tracer techniques of ^{15}N that 50% of total nitrogen was released from Anabaena and Nostoc in sixty days of incorporation in soil whereas only 26% of ^{15}N in Azolla was released during the same period. This clearly proved that Azolla is a slow nitrogen releasing fertilizer.

All these results on rice variety IR 28, conducted during summer and kharif seasons of the year, showed that Azolla improved the yield of rice grains significantly over the control.