CHAPTER II

MATERIALS AND METHODS

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1. Plant Material

The present experimental work was conducted on *Azolla pinnata* R. Br. obtained from the Gujarat Agricultural University Campus, Anand. The plants were established and maintained in a tank of the Botanical Garden of The Maharaja Sayajirao University of Baroda, Baroda.

2. Medium Preparation

Chemicals of high purity grade obtained from British Drug House (India), Sigma Chemicals Co. (U.S.A.) and E. Merck (Germany) were used in the preparation of medium. The basal medium was prepared from concentrated stock solutions which were stored at 4 °C. In the preparation of stock solutions and media, glass double distilled water was used.

The culture media viz. Johnson et al. (1966) medium, Peters and Mayne (1974 a) medium and Watanabe et al. (1977) medium were used. The composition of these media are given in the Tables 1 to 3 respectively. In the Watanabe's medium, the mineral ion concentrations work out to be as follows : K - 40 ppm, Mg - 40 ppm, Ca - 40 ppm, P - 20 ppm and Fe - 2 ppm. These are considered standard doses of metals in the medium. The supplements to be incorporated into the basal media were added before the final adjustment of the volume.

The pH of the medium was adjusted to 5.5 using either 0.1 N HCl or 0.1 N NaOH.

3. Sterilisation of media and culture vessels

Media were poured in Erlenmeyer flasks and their mouth were plugged with non-absorbent cotton. The cotton plugs were covered with brown paper to prevent absorption of moisture during sterilization. The medium was sterilized in an autoclave at a pressure of 15 p.s.i. for twenty minutes.

Gibberellic acid (GA_3) was sterilized with 0.22 μ millipore filter membrane under aseptice condition and added to the sterilized medium.

4. Aseptic techniques

All inoculations and manipulations involving axenic cultures were conducted in a Laminer flow chamber.

a. Surface sterilization :

Healthy Azolla plants were collected from the tank of the Botanical Garden. For surface sterilization of Azolla, mercuric chloride/sodium hypochlorite/hydrogen peroxide at various concentrations (0 to 10%) were used. In each case the time required for complete sterilization was adjusted. Plants were repeatedly washed with sterile distilled water before inoculation.

Table 1 : Composition of Johnson et al. (1966) medium

Constituents		Concentration in one litre of complete medium expressed as mg of hydrated salts
Potassium dihydrogen orthophosphate	KH_2PO_4	27.21
Dipotassium hydrogen phosphate	K_2HPO_4	87.58
Potassium sulphate	K_2SO_4	88.39
Calcium chloride	$CaCl_2 \cdot 2H_2O$	19.11
Calcium sulphate	$CaSO_4$	136.14
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	246.47
Boric acid	H_3BO_3	0.35
Manganese chloride	$MnCl_2 \cdot 4H_2O$	0.22
Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	0.05
Copper sulphate	$CuSO_4 \cdot 5H_2O$	0.02
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.01
Ferric citrate		
Ferric chloride	$FeCl_3$	0.69
Citric acid	$C_6H_8O_7 \cdot H_2O$	1.06
Water (double glass distilled) to make the volume of one litre. pH of the medium = 5.5		

Table 2 : Composition of Peters and Mayne (1974a) medium

Constituents		Concentration in one litre of complete medium expressed as mg of hydrated salts
Potassium chloride	KCl	149.12
Calcium chloride	$CaCl_2 \cdot 2H_2O$	294.04
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	197.17
Potassium dihydrogen orthophosphate	KH_2PO_4	54.43
Boric acid	H_3BO_3	0.28
Mangnese chloride	$MnCl_2 \cdot 4H_2O$	1.80
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.25
Copper sulphate	$CuSO_4 \cdot 5H_2O$	0.07
Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	0.21
Ferrous sulphate*	$FeSO_4 \cdot 7H_2O$	27.80
Sodium salt of ethylene diamine tetra acetic acid**	Na_2EDTA	37.22
Water (double distilled) to make the volume of one litre.		
pH of the medium = 5.5		

* The $FeSO_4 \cdot 7H_2O$ was dissolved in approximately 30 ml of double glass distilled water.

** The Na_2EDTA was dissolved in approximately 30 ml of distilled water, heated and mixed (under continuous stirring) with $FeSO_4 \cdot 7H_2O$ solution. After cooling the volume is adjusted to 100 ml. Heating and stirring results in more stable FeEDTA complex.

Table 3 : Composition of Watanabe et al. (1977) medium

Constituents		Concentration in one litre of complete medium expressed as mg of hydrated salts
Calcium chloride	$CaCl_2 \cdot 2H_2O$	146.72
Potassium sulphate	K_2SO_4	89.13
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	405.51
Sodium hydrogen orthophosphate	$NaH_2PO_4 \cdot 2H_2O$	100.73
Boric acid	H_3BO_3	1.14
Manganese chloride	$MnCl_2 \cdot 4H_2O$	1.80
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.25
Copper sulphate	$CuSO_4 \cdot 5H_2O$	0.04
Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	0.04
Ferric citrate :		
Ferric chloride	$FeCl_3$	9.62
Citric acid	$C_6H_8O_7 \cdot H_2O$	14.90
Water (double glass distilled) to make the volume of one litre. pH of the medium = 5.5		

b. Culture techniques :

Sterilized Azolla plants were inoculated in the test media. The culture flasks were incubated in the culture room at 25 ± 2 °C under fluorescent light (1000 lux) with 16/8 hours light/dark cycle.

Subculturing was done by transferring the plants to flasks containing fresh medium at regular intervals.

5. Measurement of growth

a. Fresh weight :

The plants were harvested at the end of the experimental period of three weeks and the moisture was removed from the surface with blotting paper. The fresh weights were determined on a balance.

b. Dry weight :

Weighed masses of plants were dried in an oven at 60°C and the dry weight was determined.

6. Biochemical analysis

a. Extraction and estimation of chlorophyll :

Fresh Azolla fronds were ground in pre-chilled mortar and pestle with cold acetone (80%). The extraction was repeated until the residue became colourless. The extracts were pooled together and centrifuged at 3000 rpm for 10

minutes. The supernantant was adjusted with acetone (80%) to a definite volume. Total chlorophyll content was estimated by the method as described by Yoshida et al. (1971) and expressed in mg/g fresh weight.

b. Extraction and estimation of protein :

Protein was extracted from Azolla plants by the method of Prisco and Fernandes Vieira (1976). Fresh Azolla plants were ground with 0.01 M sodium-potassium phosphate buffer (pH 7.6) containing 0.1 M sodium chloride. The ratio of tissue to the grinding medium was 1:10 (w/v). The homogenate was centrifuged at 3000 rpm for 15 minutes. From the supernantant protein was estimated by the modified Lowry's method (Hartee, 1972), using bovine serum albumin as the standard and protein content was expressed in mg/g fresh weight.

c. Estimation of total nitrogen content :

Fifty milligram of dried Azolla was digested with 5 ml of concentrated sulphuric acid in a kjeldhal flask. A pinch of copper sulphate and potassium sulphate (1:2 mixture) was added as catalyst to the digestion medium. When the solution turned colourless, its volume was adjusted with glass distilled water. The ammonium content produced was estimated using Nessler's reagent (Jackson, 1967). Ammonium sulphate was used as the standard. Nitrogen content was expressed in mg/g dry weight.

7. Measurement of nitrogenase activity by acetylene reduction

Nitrogenase catalyzed acetylene reduction activity (ARA) was measured by the procedure described by Peters and Mayne (1974b). ARA of Azolla-Anabaena complex was measured in Erlenmeyer flasks containing fresh Azolla plants and test medium (10 ml). The flasks were rendered air tight with selfsealing rubber septum, evacuated, flushed with nitrogen and then 10% acetylene was injected through the self sealing rubber septum using syringe and ethylene produced was measured on Gas Chromatograph (Chrometograph and Instruments Company, Baroda) using 2 meter Porapak N column (80-100 mesh). The column temperature and oven temperature were 95 °C and 100 °C respectively.

The ARA represented graphically on the gas chromatograph chart was quantified by measuring the height of the peak. The results were compared with standard ethylene. Acetylene reduction activity was expressed as n mole C_2H_4 formed/g fresh weight of Azolla /hr.

8. Heterocyst frequency

Individual leaves from the shoot apex of Azolla were removed separately and the symbiont Anabaena cells were released by squashing. The total number of vegetative cells and heterocysts were counted under the microscope and their sizes were also measured by using an ocular micrometer. Heterocysts were recognised due to their enlarged size, thick wall and the

presence of polar bodies from the vegetative cells of Anabaena. Heterocyst frequency was determined by the method of Hill (1975).

9. Chemical analysis

Dried Azolla samples (100 mg) were taken in nickle crucibles and they were ashed at 500 °C in a muffle furnace for two hours. The ash was collected and its mineral contents were estimated (Pocock and Vasanthy, 1986). Copper stubs pasted with double sided adhesive tape were pressed firmly into the ash for further analysis and the excess was removed by blowing with compressed air. This produces a homogenous coating of ash on the stub which was necessary for reliable analysis. Analysis was carried out using a Philips model 400 Scanning Electron Microscope provided with a computerized energy dispersive X-ray analysis system.

10. Salinity studies

The effect of salinity on Azolla pinnata has been investigated by supplementing the culture medium with various levels (5, 10, 20, 30, 40 and 50 mM) of sodium chloride (NaCl). The pH of these culture media was adjusted to 5.5. Biomass production of Azolla and nitrogenase activity were estimated under saline conditions.

a. Estimation of chlorophyll and phycocyanin from Anabaena azollae :

Chlorophyll and phycocyanin contents from individual cells of the isolated endophyte Anabaena azollae were estimated by their epifluorescence according to the method of Becking and Donze (1981). The algal cells comprising vegetative cells and heterocysts were released from the fifteenth leaf and examined under a Leitz fluorescence microscope with a fluorometer and a chart recorder. Epifluorescence of chlorophyll and phycocyanin was measured by exciting them with blue or green light from a mercury arc.

b. Extraction and estimation of the activity of ammonia assimilating enzymes :

All operations during the extraction of crude enzymes from Azolla plants were carried out at 0-4 °C.

1) Glutamine synthetase (GS) :

Glutamine synthetase (GS) activity from Azolla plants was measured by the transferase assay (Kanamori and Matsumoto, 1972). The extraction medium was 50 mM Tris. HCl (pH 7.5) containing 0.5 mM EDTA and 1.0 mM 2-mercaptoethanol. Ten percent homogenate was prepared with the extraction medium and filtered through muslin cloth. The filterate was centrifuged at 5000 rpm for 15 minutes and the supernantant was used as the enzyme source.

The assay mixture contained 0.3 ml of 0.1 M Tris. HCl (pH 7.5), 0.2 ml of 2-mercaptoethanol (1:100 v/v), 0.2 ml of 0.5 M sodium glutamate, 0.1 ml of magnesium sulphate, 0.2 ml of 0.5 M $NH_2OH.HCl$ (freshly prepared by mixing equal volume of 1.0 M NH_2OH. HCl and 1.0 M NaOH), 0.5 ml of 0.1 M ATP and 0.5 ml of crude enzyme. After 30 minutes of incubation at 37 °C, 1.0 ml of ferric chloride reagent was added (0.37 M $FeCl_3$, 0.67 N HCl and 0.2 M TCA) was added. γ-glutamyl hydroxamate formed was measured at 540 nm using Spectrophotometer (Systronic). Commercial γ-glutamyl hydroxamate was used as the standard. The enzyme activity was expressed as μ mole γ-glutamyl hydroxamate formed/mg of protein/min.

ii) Glutamate dehydrogenase (GDH) :

Glutamate dehydrogenase from Azolla plants was assayed by the method of Bulen (1956). The extraction medium contained 0.01 M Tris. HCl (pH 7.5) containing 2 mM cystein. Ten percent homogenate was prepared and filtered through muslin cloth. The filterate was centrifuged at 5000 rpm for 15 minutes and the supernantent was used as the enzyme source.

The assay mixture contained 2.1 ml of Tris. HCl (pH 7.5), 0.1 ml of 3 M NH_4Cl, 0.1 ml of 0.3 M ∝ ketogluteric acid, 0.2 ml of 1.0 mM NADPH and 0.5 ml of crude enzyme. The enzyme activity was measured by the oxidation of NADPH at 340 nm using Spectrophotometer (Shimatzu).

iii) Glutamate synthase (GOGAT) :

Glutamate synthase activity from Azolla plants was assayed by Sodek and Dasilva (1977) method. Extraction medium contained 50 mM Tris. HCl (pH 7.5) containing 1.0 mM EDTA and 10 mM 2-mercaptoethanol. Ten percent homogenate was prepared and filtered through muslin cloth. The filterate was centrifuged at 5000 rpm for 15 minutes. The supernantant was used as the enzyme source.

The assey mixture consisted 1.2 ml of 1.0 M Tris. HCl (pH 7.5), 0.2 ml of 0.2 M α ketoglutaric acid, 0.2 ml of 0.2 M glutamine, 0.2 ml of 2-mercaptoethanol (1:100 v/v), 0.3 ml of 0.1 M KCl, 0.2 ml of 0.2 M EDTA, 0.2 ml of 1.0 mM NADPH and 0.5 ml of crude enzyme. The enzyme activity was measured by the oxidation of NADPH at 340 nm.

11. Anatomical studies

Individual leaves of Azolla plants from its apex were serially numbered. Shoot apex and each leaf upto 22nd leaf were excised and fixed in CRAF III reagent, dehydrated by passing through alcohol : xylene series and ultimately embeded in paraffin wax. Serial sections were cut at 10 μ on a rotary microtome. Staining was done with saffranin "O". After usual dehydration through ethyl alcohol and xylene, the sections were mounted in DPX (Jensen, 1962).

12. Ammonification of Azolla nitrogen

Two grams of fresh Azolla in its vegetative stage of development and in its sporulating stage were mixed separately with 20 g of soil from rice field. The mixture was flooded with distilled water and incubated in dark at 30 °C. Ammonia released from the decomposing Azolla was extracted periodically according to the method described by Watanabe et al. (1977) and estimated according to the method described by Jackson (1967).

13. Application of Azolla and/or inorganic nitrogen fertilizer to rice variety IR 28 :

The effect of Azolla and/or inorganic nitrogen fertilizer on the performance of rice variety IR 28 was determined during summer season (date of transplantation : 4-2-86, date of harvest : 22-5-86) and kharif season (date of transplantation: 3-9-86, date of harvest : 7-12-86) of the year 1986. P_2O_5 at the rate of 40 kg/ha basis as superphosphate and K_2O at the rate of 40 kg/ha basis as muriate of potash were applied to all the pots. The following treatments were given :

1. Incorporation of Azolla (10 t /ha in two splits)
2. 80 kg N/ha as ammonium sulphate (in two splits)
3. Incorporation of Azolla (10 t /ha in two splits)+ 80 kg N/ha basis as ammonium sulphate (in two splits)
4. Control received no treatment

The first split of Azolla and/or inorganic nitrogen fertilizer was added to the soil at the time of transplantation and second split after one month of transplantation. The rice variety IR 28 seedlings (25 days old) were transferred to the pots. Two hills per pot were grown. Regular plant protection measures and cultural practices were adopted. The following biometrical observations were made :

a. Plant height :

The height of rice plants on 30th, 60th and 90th day after transplantation were measured from the soil surface to the tip of the tallest tiller.

b. Shoot and root weight :

Rice plants were collected at regular intervals and washed thoroughly under running tap water. After blotting them, the shoots and roots were separated and their fresh weights were taken. These shoots and roots were dried at 60 °C to a constant weight and the dry weight of shoots and roots were recorded.

c. Nitrogen content of shoot and root :

Total nitrogen contents from dried shoot and root were estimated by the method described by Jackson (1967).

d. Tiller number :

The number of tillers from rice plants were counted and recorded.

e. Panicle length :

Length of the panicle was measured from the last internode of the spike to the tip. Mean of five panicles from the same hill was considered.

f. Grain and straw yield :

Individual hill in each treatment were harvested separately and the straw and grain yield were recorded.

g. Hundred grains weight :

The weight of hundred grains from each treatments was recorded.

14. Photography

Photographs were taken using ORWO 22 black and white negatives and Konica coloured negatives. Photomicrography was done using Carl Zeiss microscope and Leitz fluorescence microscope.

15. Statistical analysis

Experimental results were statistically analysed and critical difference (C.D.) at 5% probability level was

calculated. Correlation coefficient (r) between variables and "t" test were done as per the procedures described by Dospekhov (1979).