CHAPTER III

IN VITRO EXPERIMENTAL STUDIES ON *AZOLLA PINNATA* R.Br.

IN VITRO EXPERIMENTAL STUDIES ON AZOLLA PINNATA R.Br.

Azolla is a free floating aquatic fern living in symbiotic association with Anabaena azollae. Since the efficiency of Azolla as a biofertilizer depends upon its biomass production at a faster rate accompanied with increased nitrogen fixing ability, studies concerning the nutritional/hormonal requirements have been conducted on Azolla pinnata R.Br., the common Indian species.

In addition the anatomical details and the effects of sodium chloride-induced salinity on A. pinnata were explored. Stock cultures of A. pinnata were maintained in a tank in the Botanical Garden of the M.S. University of Baroda, Baroda.

This chapter deals with the results of the experimental work conducted on A. pinnata under in vitro conditions which are described in the following sections :

SECTION A : Nutritional studies

Experiment 1

Establishment of axenic stock cultures of Azolla pinnata R.Br.

Healthy Azolla plants collected from the tank (Plate 1) were washed under running tap water and finally rinsed with distilled water.

Plate 1 *Azolla pinnata* R. Br. in a tank in the Botanical Garden of the M.S. University of Baroda.

Plate 1

Three surface sterilizing agents viz. mercuric chloride (0.05, 0.1, 0.2, 0.3% w/v), sodium hypochlorite (0.05, 0.1, 0.2% v/v) and hydrogen peroxide (5, 10 and 15% v/v) were used. One gram each of healthy Azolla plants were surface sterilized in each of the reagents in the given concentrations. The plants were maintained with constant shaking in 150 ml Erlenmeyer Flasks for 1, 2, 3, 4 and 5 minutes each, in each of the given concentrations. They were washed with sterile distilled water several times and inoculated in nitrogen free Watanabe et al. (1977) medium (pH 5.5). Culture flasks were incubated at 25 ± 2°C in culture room with cool, white fluorescent light (1000 Lux) for a 16/8 hours light/dark cycle.

Both mercuric chloride and sodium hypochlorite used as surface sterilizing agents were found to be toxic for Azolla plants in all the concentrations and duration of times specified.

Azolla plants treated with 5% hydrogen peroxide were found to be contaminated after one week of culture period while those treated with 10% hydrogen peroxide for three minutes were free of contamination. After a culture period of three days nearly 60% of these surviving Azolla plants were healthy in appearance. They were transferred to fresh nitrogen free Watanabe medium, as described in Chapter II, Materials and Methods (4b). These plants picked up growth

and development due to which new plants were produced covering the entire surface of the medium. Within eight weeks of culture period, a number of axenic stock cultures were thus initiated (Plate 2). It was observed that 15% (v/v) hydrogen peroxide was toxic to Azolla and was therefore rejected.

Experiment 2

Effect of renewal of culture medium on biomass production of A. pinnata

It was observed in Experiment No.1 that stock culture of Azolla turned yellow after a week. Therefore the effect of periodical renewal of the culture medium on biomass production was studied to understand if that would prevent the yellowing of the plants.

Equal quantity of healthy Azolla plants (300 ± 20 mg) from stock cultures were transferred to nitrogen free Watanabe medium (50 ml), pH 5.5. Culture flasks were incubated at 25 ± 2°C in culture room and illuminated with cool, white fluorescent light (1000 Lux) for 16/8 hours light/dark cycle. Two such sets were prepared. In one set (Set I), regular weekly subculturing of the plants in fresh medium was carried out while in the other set (Set II) the plants were allowed to grow for the entire period of three weeks without renewing the medium. Plants were harvested from both the sets at

Plate 2 Axenic culture of *A. pinnata*

Plate 2

weekly intervals, and their biomass calculated and recorded (Table 4). The pH of the conditioned medium was also measured at each week. At the end of first week, a 2.4-fold increase in biomass of Azolla in terms of fresh and a 2.1-fold in terms of dry weights were observed in both the sets and the pH of the medium was decreased from 5.5 to 5.17.

At the end of second and third week, Azolla in response to renewal of medium in set I an increase of 7-fold in biomass production with no change in pH was recorded (Table 4).

On the other hand, harvest from the continued growth in the same medium at the end of the third week (Set II), showed only a 3.7-fold increase in biomass of Azolla. The pH tended to decrease at each week.

Therefore, it was found beneficial to weekly renew the culture medium of Azolla. Since with three weeks of growth, the entire surface of the medium was sufficiently covered by Azolla plants, the experimental period was not extended further.

Experiment 3

Selection of suitable culture medium for Azolla

For maximum biomass production of Azolla, three known nitrogen free culture media viz. Johnson et al. (1966),

Table 4 : Effect of renewal of culture medium on biomass production of *Azolla pinnata* R. Br.

Inoculum : Fresh wt. = 300 ± 20 mg
Dry wt. = 13 ± 01 mg

Weekly observations	Set I Medium renewed	Set II Medium not renewed
First		
Fresh weight (mg)	730 ± 32	730 ± 32
Dry weight (mg)	28 ± 3	28 ± 3
pH of the medium	5.17	5.17
Second		
Fresh weight (mg)	1310 ± 48	980 ± 39
Dry weight (mg)	52 ± 7	39 ± 4
pH of the medium	5.16	4.93
Third		
Fresh weight (mg)	2080 ± 62	1130 ± 34
Dry weight (mg)	82 ± 11	48 ± 6
pH of the medium	5.19	4.31

Mean of six replicates with S.D.

Peters and Mayne (1974,a) and Watanabe et al. (1977) were tested. The pH of the medium was maintained at 5.5. Azolla plants about 300 ± 20 mg from the axenic stock cultures were transferred to each of the culture medium (50 ml). Weekly renewal of the culture media was done, and biomass was harvested after three weeks period.

Table 5, gives the data about fresh and dry weights of Azolla plants grown in different media. Highest biomass production being 7-fold of the original has recorded in Watanabe medium. The next best was Peters and Mayne's followed by Johnsons media. Further experiments were therefore, carried out using Watanabe medium.

Experiment 4

Effect of pH of Watanabe medium on growth, composition and acetylene reduction activity of A. pinnata

In the present experiment, the optimal pH of the Watanabe medium which would support the highest biomass production of Azolla and the highest nitrogenase activity as measured by acetylene reduction method have been studied. Healthy Azolla plants (300 ± 20 mg) from axenic stock culture were transferred to nitrogen free Watanabe medium (50 ml) at various pH levels (4.5, 5.5, 6.5, 7.5 and 8.5). Cultural conditions as described in Chapter II, Materials and Methods (4b) have been followed. After three weeks of experimental

Table 5 : Biomass production of *Azolla* in different culture media

Inoculum : Fresh wt. = 300 ± 20 mg
Dry wt. = 13 ± 01 mg

Biomass	Johnson's medium	Peters and Mayne's medium	Watanabe's medium
Fresh weight (mg)	1860±31	2010±4.2	2075±18
Dry weight (mg)	78±7	84±9	88±4

Mean of six replicates with S.D.

period, Azolla plants were harvested and their fresh and dry weights were recorded. Chlorophyll, protein, nitrogen contents, nitrogenase activity measured by acelytene reduction assay and heterocyst frequency were estimated as described in Chapter, Materials and Methods (6a, b and c).

Azolla plants cultured in Watanabe medium at pH 4.5 turned pale green in colour and the roots became brown. Plants grown at pH 5.5 and 6.5 were dark green with well developed roots. At pH 7.5 and 8.5, the growth of Azolla plants declined, its multiplication decreased and the plants remained small in size.

At pH 5.5 and 6.5, the fresh and dry weights of biomass of Azolla plants, both these and other parameters viz. chlorophyll content, protein content, acetylene reduction activity and heterocyst frequency were significantly higher than that observed at 4.5, 7.5 and 8.5 (Table 6).

Therefore from the range of pH tried, 5.5 to 6.5 was found to be suitable for Azolla culture under controlled conditions. In all further experiments, pH of the medium was kept at 5.5.

Table 6 : Influence of pH on the growth, chlorophyll, protein, nitrogen contents, acetylene reduction activity and heterocyst frequency of *A. pinnata*

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ± 01 mg

pH levels	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
4.5	1520	64	0.41	24.1	3.7	590	27
5.5	2095	89	0.52	24.8	3.9	672	28
6.5	1920	81	0.49	24.6	3.9	638	28
7.5	1685	71	0.41	23.9	3.8	578	26
8.5	1410	58	0.39	23.6	3.7	540	24
C.D. at 5%	128	9	0.10	0.4	0.9	58	0.9

Mean of six replicates

Experiment 5

Effect of various levels of mineral nutrients in Watanabe medium on growth, composition and nitrogenase activity of A. pinnata

Watanabe medium was prepared with each of the following mineral nutrients in concentrations of 0, 10, 20, 30, 40 and 50ppm : Potassium (K), Magnesium (Mg), Phosphorous (P), Calcium (Ca) while Iron (Fe) concentrations were 0, 1, 2 and 3 ppm.

Azolla plants (300 ± 20 mg) from axenic stock cultures were transferred to each of the test medium (50 ml in 150 ml Erlenmeyer flask) containing different levels of mineral nutrient, making sure that carry over effects were removed.

After three week period, plants were harvested and their fresh and dry weights recorded. Chlorophyll, protein, nitrogen contents, nitrogenase activity measured by acetylene reduction assay and heterocysts frequency were estimated as described in Chapter II, Materials and Methods.

a) Potassium (K)

Effect on biomass

In complete absence of K and at 10 ppm K levels, negligible growth or multiplication occurred and chlorosis appeared on the leaves (Plate 3). With the increase in K

Plate 3 Effects of K levels (a=0, b=10, c=20, d=30, e=40 and f=50 ppm) in Watanabe medium on *A. pinnata*

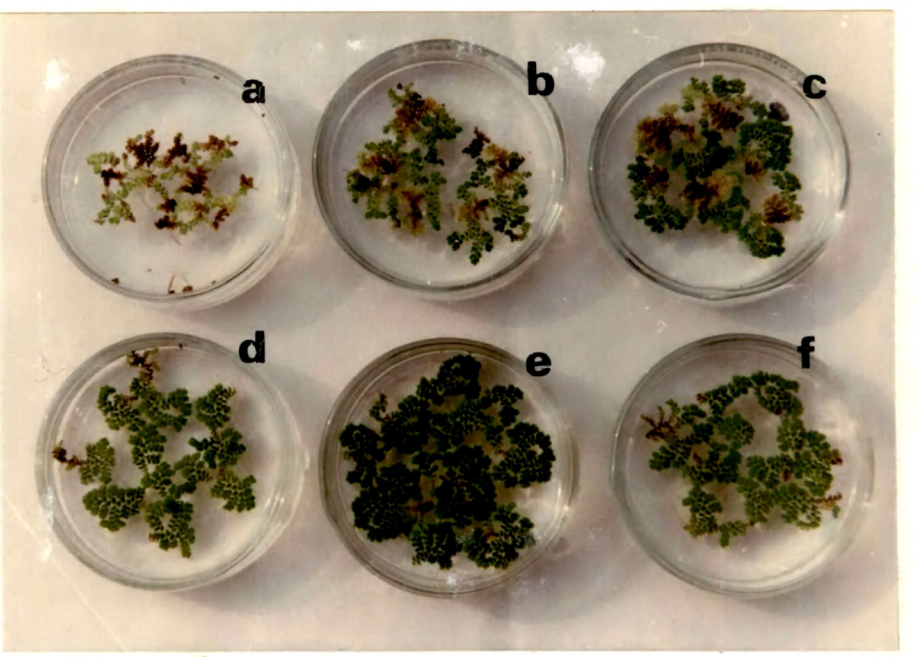

Plate 3

level to 20 and 30 ppm, the plants showed growth and were green in colour with short roots. At the standard dose of 40 ppm of K, Azolla plants were dark green with well developed roots. With further increase in K level to 50 ppm, the plants decreased in their growth and remained small in size as compared to the standard. At 10, 20, 30, 40 ppm of K levels, linear increase in the biomass production occurred (Table 7). However, the biomass production was significantly higher at the standard dose of 40 ppm and at 50 ppm of K, than recorded at the lower doses (Fig. 1).

Effect on composition

Even chlorophyll and protein contents were significantly higher at 40 and 50 ppm as compared to the rest of the treatments. Nitrogen content was though not significantly increased but was highest at 40 ppm K level.

Effect on nitrogenase activity

In the complete absence of K, 60% reduction in acetylene reduction activity along with 33% reduction in heterocyst frequency in Anabaena of Azolla has been recorded, when compared with the Azolla plants cultured in standerd level of K in the culture medium. Data indicated that the K concentrations of 40 and 50 ppm were significantly superior with respect to nitrogenase activity as compared to the rest of the concentrations. Considering all parameters

Table 7 : Effect of various levels of potassium (K) on the growth, composition, acetylene reduction activity and heterocyst frequency of *A. pinnata*

Inoculum : Fresh weight = 300 ± 20 mg Dry wt. = 13 ± 01 mg

K-levels (ppm)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
0	790	33	0.37	17.9	2.9	278	20
10	940	40	0.41	19.1	3.1	368	21
20	1510	65	0.47	20.3	3.2	432	24
30	1710	76	0.49	21.7	3.5	586	26
40 (standard)	2080	89	0.52	24.3	3.9	690	30
50	2070	87	0.52	24.1	3.8	687	29
C.D. at 5%	98	6.7	0.02	1.3	0.9	76	1.4

Mean of six replicates

Fig. 1 Effects of K levels (1=0, 2=10, 3=20, 4=30 and 5 = 50 ppm) in the Watanabe medium on dry weight, protein content, ARA, chlorophyll content, nitrogen content and heterocyst frequency of *A. pinnata*, after a period of three weeks.

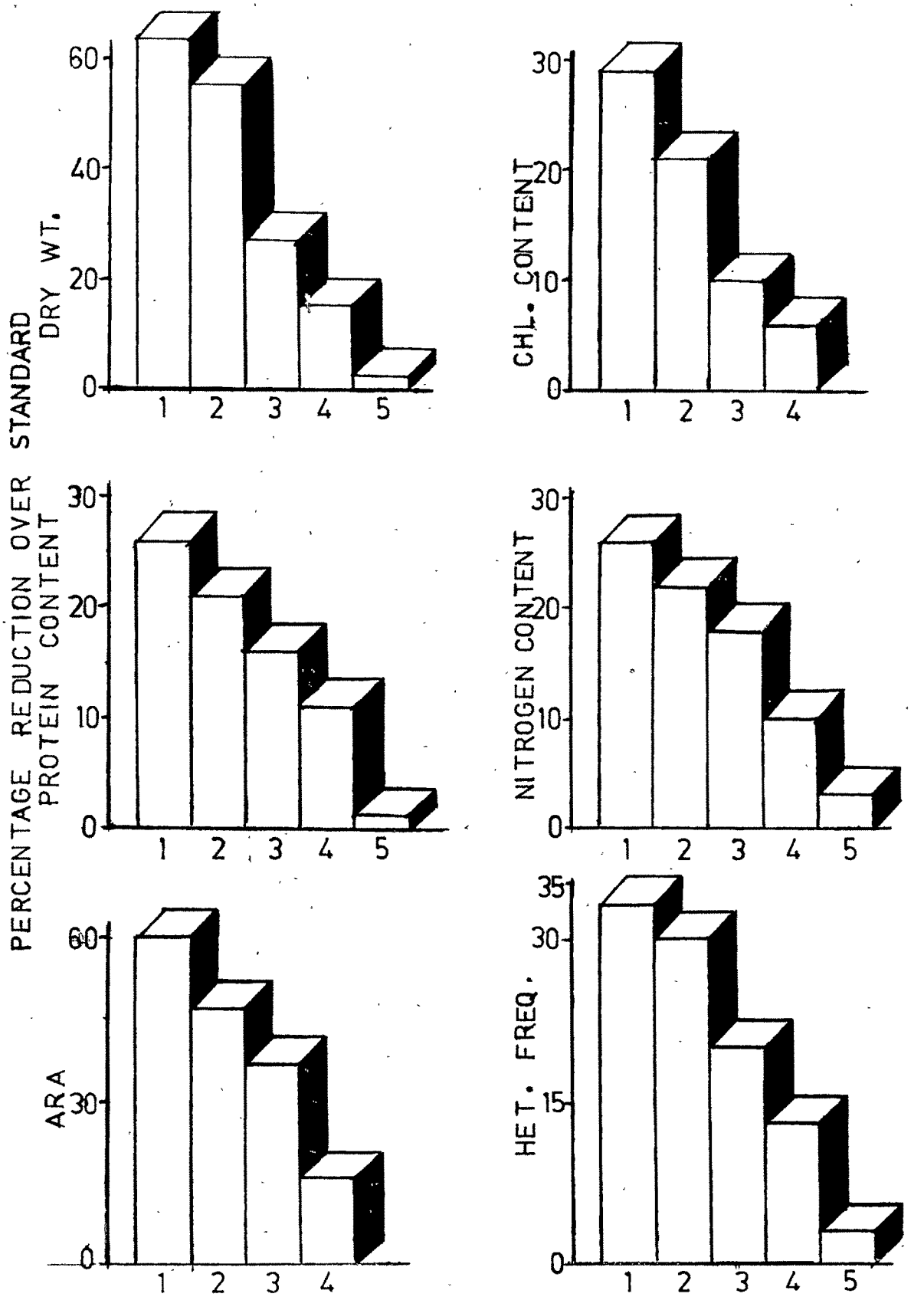
STANDARD DRY WT.
60
40
20
0
1 2 3 4 5
CHL. CONTENT
30
20
10
0
1 2 3 4
PERCENTAGE REDUCTION OVER PROTEIN CONTENT
30
20
10
0
1 2 3 4 5
NITROGEN CONTENT
30
20
10
0
1 2 3 4 5
ARA
60
30
0
1 2 3 4
HET. FREQ.
35
30
15
0
1 2 3 4 5

Fig. 1

40 ppm K level even had the edge over 50 ppm which proved that 40 ppm K level in the standard Watanabe medium was the most optimum level in all respects.

Positive correlation was observed between biomass production (fresh weight) and acetylene reduction activity ($r = 0.975$), and between nitrogen content accumulated in dry matter ($r = 0.954$) in response to various K levels in the medium.

b) Magnesium (Mg)

Effect on biomass

Complete elimination as well as at lower level of Mg (10 and 20 ppm) caused chlorosis upon the leaves. Chlorosis initially was noticed upon the older and later on the younger leaves (Plate 4). Roots got detached from Azolla plants within five days of culturing. Absence of Mg reduced 67% biomass production when compared with plants cultured in standard dose of Mg (40 ppm). At 30 ppm of Mg, the biomass production improved and reached to an optimal level at 40 ppm of Mg in the culture medium. Plants cultured in standard level of Mg (40 ppm) were dark green with well developed roots. Further increase in Mg concentration (50 ppm) did not improve biomass production of Azolla (Table 8).

Plate 4 Effects of Mg levels (a=0, b=10, c=20, d = 30, e=40 and f=50 ppm) in Watanabe medium on *A. pinnata*.

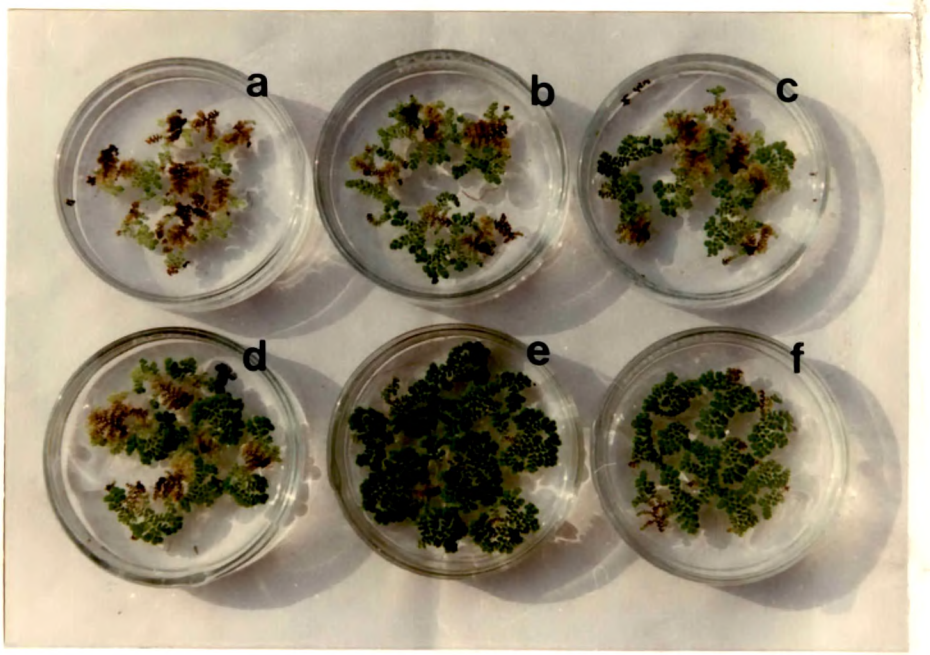

Plate 4

Table 8 : Effect of various levels of magnesium (Mg) on the growth, composition, acetylene reduction activity and heterocyst frequency of A. pinnata

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ± 01 mg

Mg-levels (ppm)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
0	710	29	0.17	15.2	2.4	178	21
10	925	39	0.29	16.9	2.7	234	23
20	1210	51	0.41	18.1	2.9	376	24
30	1790	75	0.47	19.4	3.2	510	26
40 (standard)	2065	87	0.52	22.3	3.7	680	29
50	1970	83	0.51	22.1	3.6	671	29
C.D. at 5%	180	8.6	0.02	2.1	0.4	142	2.4

Mean of six replicates

Effect on composition

In complete absence of Mg, 67% reduction in chlorophyll content, 32% and 35% in protein and nitrogen contents respectively, occurred compared with the values of Azolla plants cultured in standard level of Mg (40 ppm) (Fig. 2). The addition of Mg at 10 to 50 ppm improved the Azolla composition while at 40 ppm of Mg, Azolla exhibited optimal levels of chlorophyll, protein and nitrogen contents.

Effect on nitrogenase activity

In complete absence of Mg in culture medium 28% heterocyst frequency was reduced with corresponding reduction to 74% in ARA when compared with that of the Azolla plants cultured in standard medium. Thus the presence of standard dose of Mg (40 ppm) was found to be essential for Azolla growth. It was noticed that 40 and 50 ppm concentrations of Mg yielded significantly higher fresh and dry biomass; chlorophyll, protein and nitrogen contents and also increased ARA and heterocyst frequency; compared to the rest of the levels of Mg tested. Numerically 40 ppm was better compared to the higher level i.e. 50 ppm of Mg.

A positive correlation was found to exist between biomass production of Azolla and its ARA ($r = 0.987$). Thus 40 ppm of Mg that was normally incorporated in the standard Watanabe medium was proved to be the optimal level.

Fig. 2 Effects of Mg levels (1=0, 2=10, 3=20, 4=30 and 5=50) in the Watanabe medium on dry weight, protein content, ARA, chlorophyll content, nitrogen content and heterocyst frequency of *A. pinnata*, after a period of three weeks.

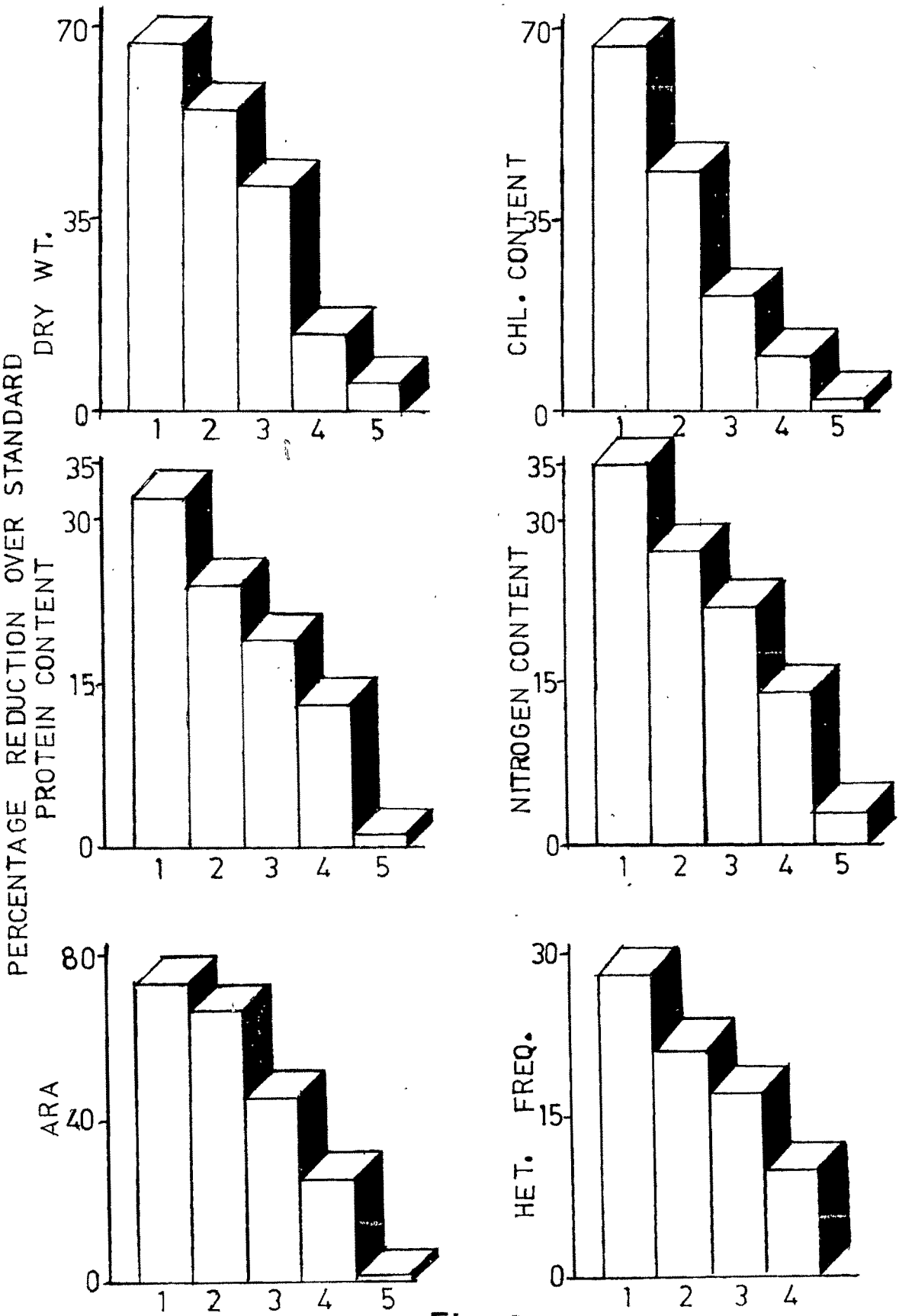
PERCENTAGE REDUCTION OVER STANDARD
DRY WT.
70
35
0
1 2 3 4 5
CHL. CONTENT
70
35
0
1 2 3 4 5
PROTEIN CONTENT
35
30
15
0
1 2 3 4 5
NITROGEN CONTENT
35
30
15
0
1 2 3 4 5
ARA
80
40
0
1 2 3 4 5
HET. FREQ.
30
15
0
1 2 3 4

Fig. 2

c) Calcium (Ca)

Effect on biomass

In complete absence and at 10 ppm of Ca in Watanabe medium, Azolla plants reduced growth and remained small in size. Roots were not much developed in these plants (Plate 5). At 20 and 30 ppm of Ca levels, plants were green in colour with short roots. As the Ca level in the culture medium reached 40 ppm (the standard dose) the Azolla plants were found to be dark green with well developed roots. Increase in Ca level to 50 ppm, did not improve Azolla growth any further. In absence of Ca, biomass production of Azolla was reduced to 74%, when compared with Azolla plants cultured in medium containing standard dose of Ca. With addition of Ca, the biomass production of Azolla was improved, ultimately reached its optimal level when Ca was 40 ppm in the culture medium (Table 9).

Effect on composition

Absence of Ca in culture medium reduced to 48%, 27% and 26% the chlorophyll, protein and nitrogen contents respectively of Azolla as compared with those values obtained when Azolla cultured in medium of standard dose of Ca (Fig. 3).

Plate 5 Effects of Ca levels (a=0, b=10, c=20, d=30, e=40 and f=50 ppm) in Watenabe medium on *A. pinnata*.

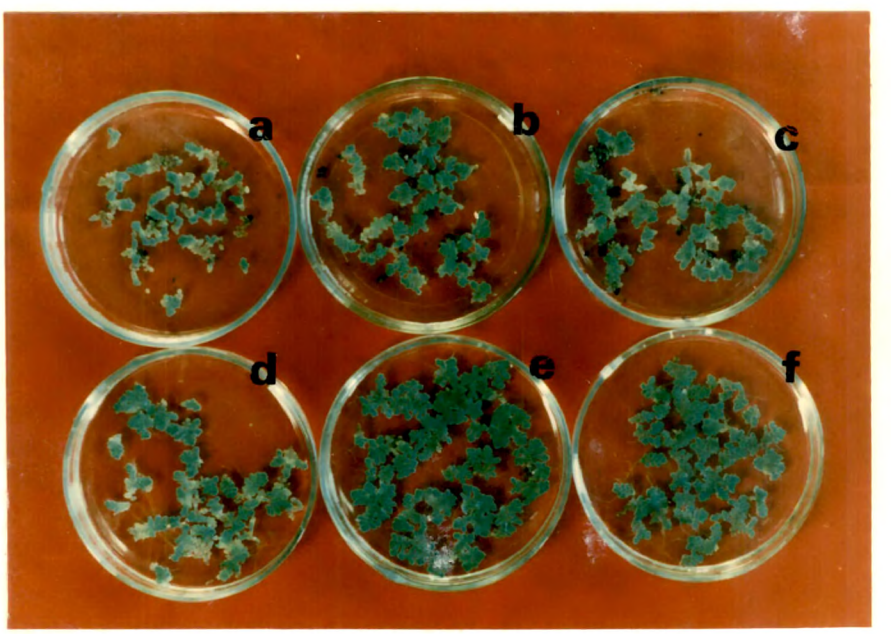

Plate 5

Table 9 : Effect of various levels of calcium (Ca) on the growth, composition, acetylene reduction activity and heterocyst frequency of *A. pinnata*

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ±01 mg

Ca-levels (ppm)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
0	540	23	0.27	17.9	2.9	139	19
10	920	39	0.31	19.8	3.2	276	21
20	1170	50	0.39	21.7	3.4	398	25
30	1820	77	0.49	23.9	3.8	512	26
40 (standard)	2075	88	0.52	24.6	3.9	660	28
50	2050	87	0.51	24.2	3.8	648	28
C.D. at 5%	128	9.8	0.03	1.7	0.9	98	1.7

Mean of six replicates

Fig. 3 Effects of Ca levels (1=0, 2=10, 3=20, 4=30 and 5=50 ppm) in the Watanabe medium on dry weight, protein content, ARA, chlorophyll content, nitrogen content and heterocyst frequency of A. pinnata, after a period of three weeks.

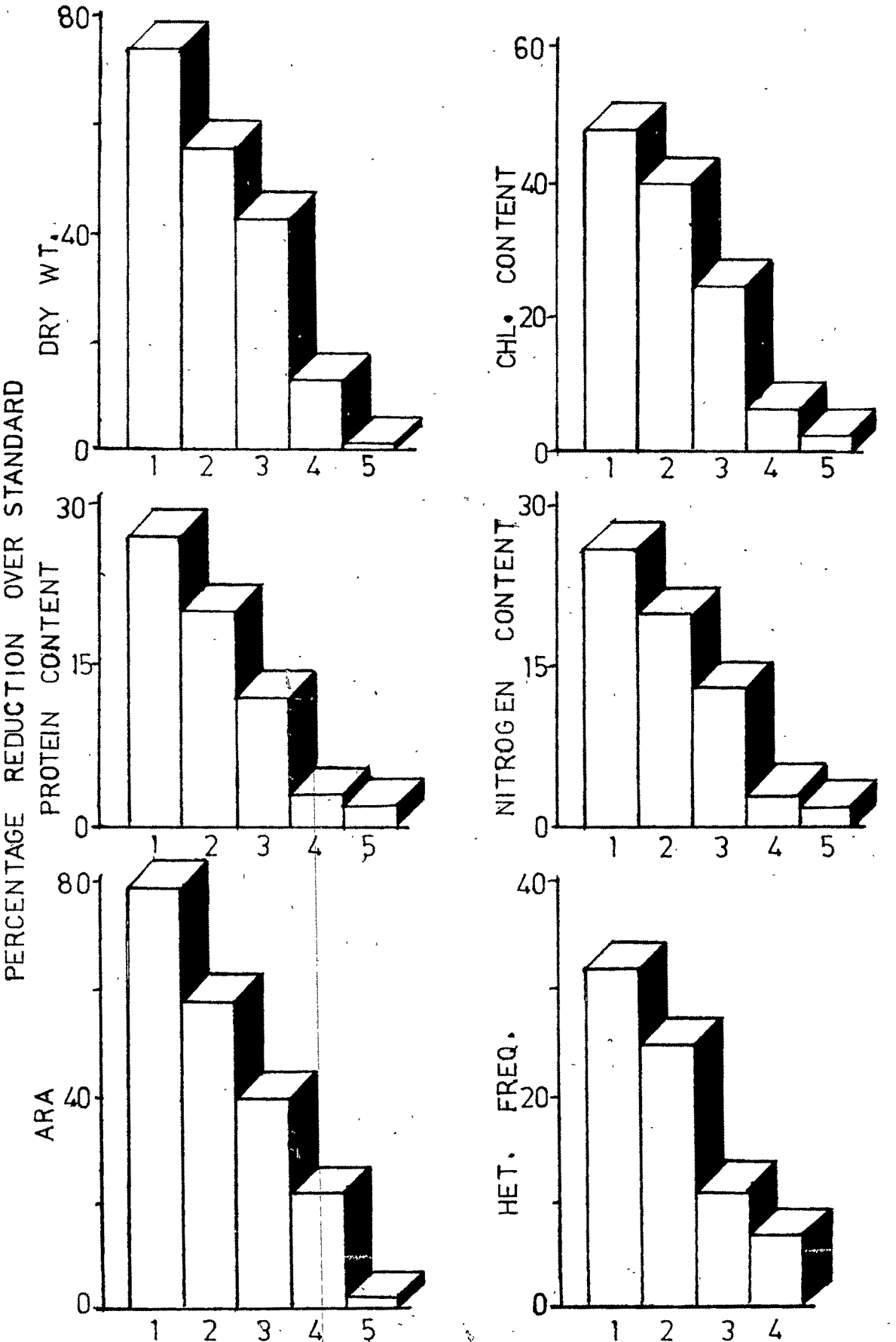
PERCENTAGE REDUCTION OVER STANDARD
DRY WT.
80
40
0
1 2 3 4 5
CHL. CONTENT
60
40
20
0
1 2 3 4 5
PROTEIN CONTENT
30
15
0
1 2 3 4 5
NITROGEN CONTENT
30
15
0
1 2 3 4 5
ARA
80
40
0
1 2 3 4 5
HET. FREQ.
40
20
0
1 2 3 4

Fig. 3

Effect on nitrogenase activity

Absence of Ca in the medium reduced heterocyst frequency by 32% and acetylene reduction activity to 79%. The gradual addition of Ca to the culture medium improved both these growth parameters, both reaching to optimal levels at 40 ppm of Ca in the medium.

A positive correlation existed between biomass production of Azolla and its nitrogenase activity (r = 0.990). Besides, nitrogen content exhibited a positive correlation with biomass produced (r = 0.988). Hence, a concentration of 40 ppm Ca was found to be the optimal concentration for A. pinnata biomass production.

d) Phosphorus (P)

Effect on biomass

The visible symptoms of phosphorus deficiency were reduced growth, the fronds remaining small, pale green in colour and roots turned brown (Plate 6). Reduction in growth was 80% when compared with Azolla plants grown in standard medium (Fig. 4). At 10 and 20 ppm of P, there was an improvement in biomass production but the highest biomass production was recorded in a medium conteining 30 ppm P. Actually 30 ppm, P was higher than the level of P (20 ppm) present in standard medium. Further increase in P level to 40 ppm did not improve biomass production of Azolla.

Plate 6 Effects of P levels (a=0, b=5, c=10, d=20, e=30 and f=40 ppm) in Watanabe medium on *A. pinnata*

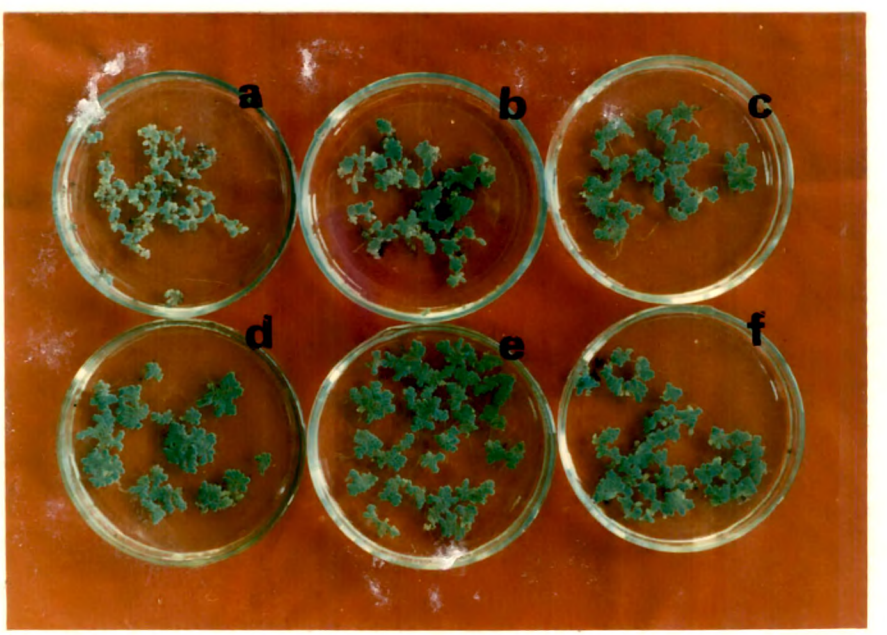

Plate 6

Fig. 4 Effects of P levels (1=0, 2=5, 3=10, 4=30 and 5=40 ppm) in the Watanabe medium on dry weight, protein content, ARA, chlorophyll content, nitrogen content and heterocyst frequency of A. pinnata, after a period of three weeks.

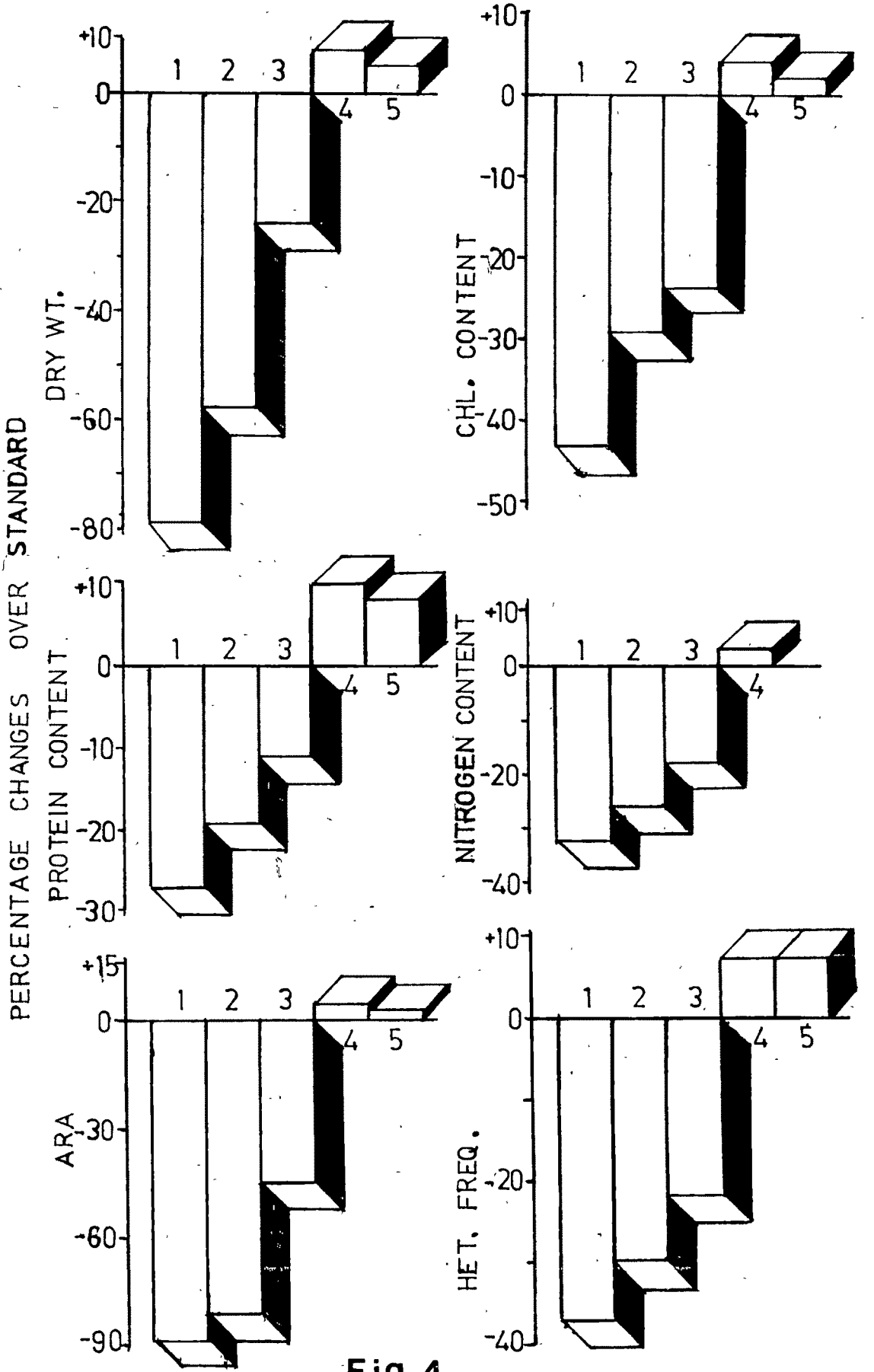
PERCENTAGE CHANGES OVER STANDARD
DRY WT.
+10
0
-20
-40
-60
-80
1 2 3 4 5
CHL. CONTENT
+10
0
-10
-20
-30
-40
-50
1 2 3 4 5
PROTEIN CONTENT
+10
0
-10
-20
-30
1 2 3 4 5
NITROGEN CONTENT
+10
0
-20
-40
1 2 3 4
ARA
+15
0
-30
-60
-90
1 2 3 4 5
HET. FREQ.
+10
0
-20
-40
1 2 3 4 5

Fig. 4

Table 10 : Effect of various levels of phosphorus (P) on the growth, composition, acetylene reduction activity and heterocyst frequency of *A. pinnata*

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ± 01 mg

P- levels (ppm)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr. wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
0	420	18	0.29	16.1	2.6	72	17
5	930	36	0.36	17.8	2.8	198	19
10	1570	65	0.39	19.7	3.1	370	21
20 (standard)	2090	85	0.50	22.1	3.8	673	27
30	2230	92	0.53	24.3	3.8	710	29
40	2170	89	0.52	23.8	3.8	698	29
C.D. at 5%	118	5.1	0.02	1.7	0.7	42	1.3

Mean of six replicates

Effect on composition

Azolla cultured in complete absence of P showed 42% and 27% reductions in chlorophyll, protein and nitrogen contents respectively. At 30 ppm of P level, which was higher than that present in the standard medium (20 ppm) supported an optimal chlorophyll and nitrogen contents. Further increase to 40 ppm of P was not effective in improving the composition of A. pinnata.

Effect on nitrogenase activity

Heterocyst frequency in Anabaena azollae was reduced to 37% and acetylene reduction activity to 89% when Azolla was grown in complete absence of P. At 30 ppm of P, the heterocyst frequency and acetylene reduction activity were found to be optimal. Actually, about 7% increase in heterocyst frequency and acetylene reduction activity were recorded at 30 ppm level as compared to Azolla plants cultured at standard dose of 20 ppm of P.

The present studies on P nutrient of Azolla plants showed that positive correlation existed between biomass production and nitrogenase activity ($r = 0.986$). Also nitrogen content accumulated showed positive correlation with dry matter ($r = 0.972$).

Hence, a level of 30 ppm P which was slightly higher than present in Watanabe medium, was found to be optimal

level for the Indian species of Azolla tested when cultured under controlled conditions.

e) Iron (Fe)

Effect on biomass

Azolla plants showed fragmentation in complete absence of Fe in the culture medium. Plants showed extreme chlorosis and roots remained short and were of brown colour (Plate 7). Azolla cultured in 2 ppm Fe containing medium showed dark green fronds with well developed roots.

The biomass production was reduced to 73% when compared with plants grown in standard dose, 2 ppm of Fe. Nearly 7-fold increase in biomass production was recorded at 2 ppm of Fe level. At 3 ppm level of Fe, there was no further increase in biomass production (Table 11).

Effect on composition

Chlorophyll content was reduced to 33%, in complete absence of Fe in the medium. Protein and nitrogen contents were also reduced. At 2 ppm Fe level, all these three growth parameters were at their optimal levels (Fig. 5).

Effect on nitrogenase activity

Heterocyst frequency showed reduction in the absence of Fe which affected nitrogenase activity. A positive

Plate 7 Effects of Fe levels (a=0, b=1, c=2 and d=3 ppm) in Watanabe medium on *A. pinnata*

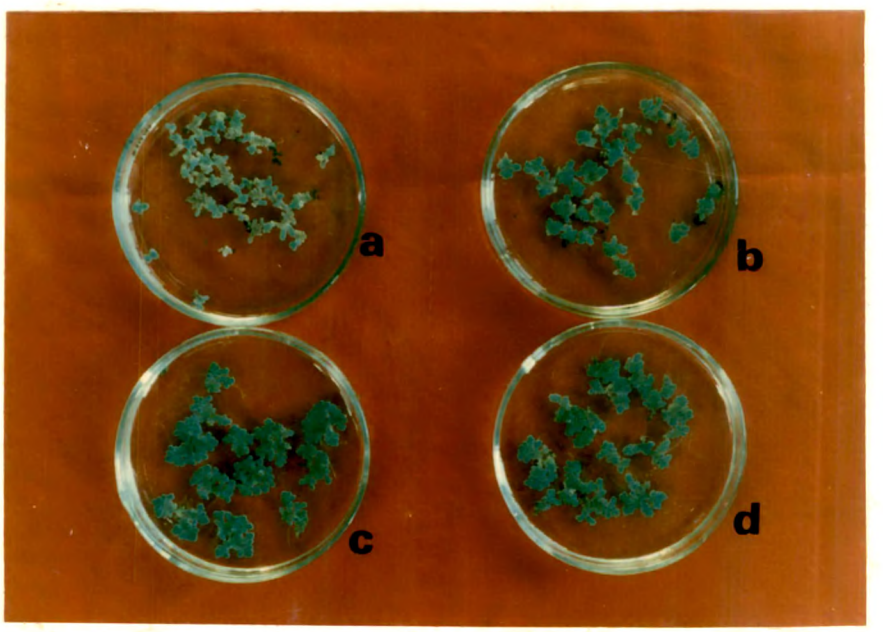

Plate 7

Table 11 : Effect of various levels of iron (Fe) on the growth, composition, acetylene reduction activity and heterocyst frequency of *A. pinnata*

Inoculum : Fresh wt. 300 ± 20 mg Dry wt. = 13 ± 01 mg

Fe-levels (ppm)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
.0	620	23	0.36	19.1	2.8	32	21.1
1.0	1170	49	0.46	24.3	3.5	510	24.7
2.0 (standard)	2090	87	0.54	26.8	3.9	658	28.3
3.0	2060	86	0.53	26.7	3.8	650	28.3
C.D. at 5%	260	19	0.09	1.8	0.3	127	1.3

Mean of six replicates

Fig. 5 Effects of Fe levels (1=0, 2=1, and 3=3 ppm) in the Watanabe medium on dry weight, protein content, ARA, chlorophyll content, nitrogen content and heterocyst frequency of *A. pinnata*, after a period of three weeks.

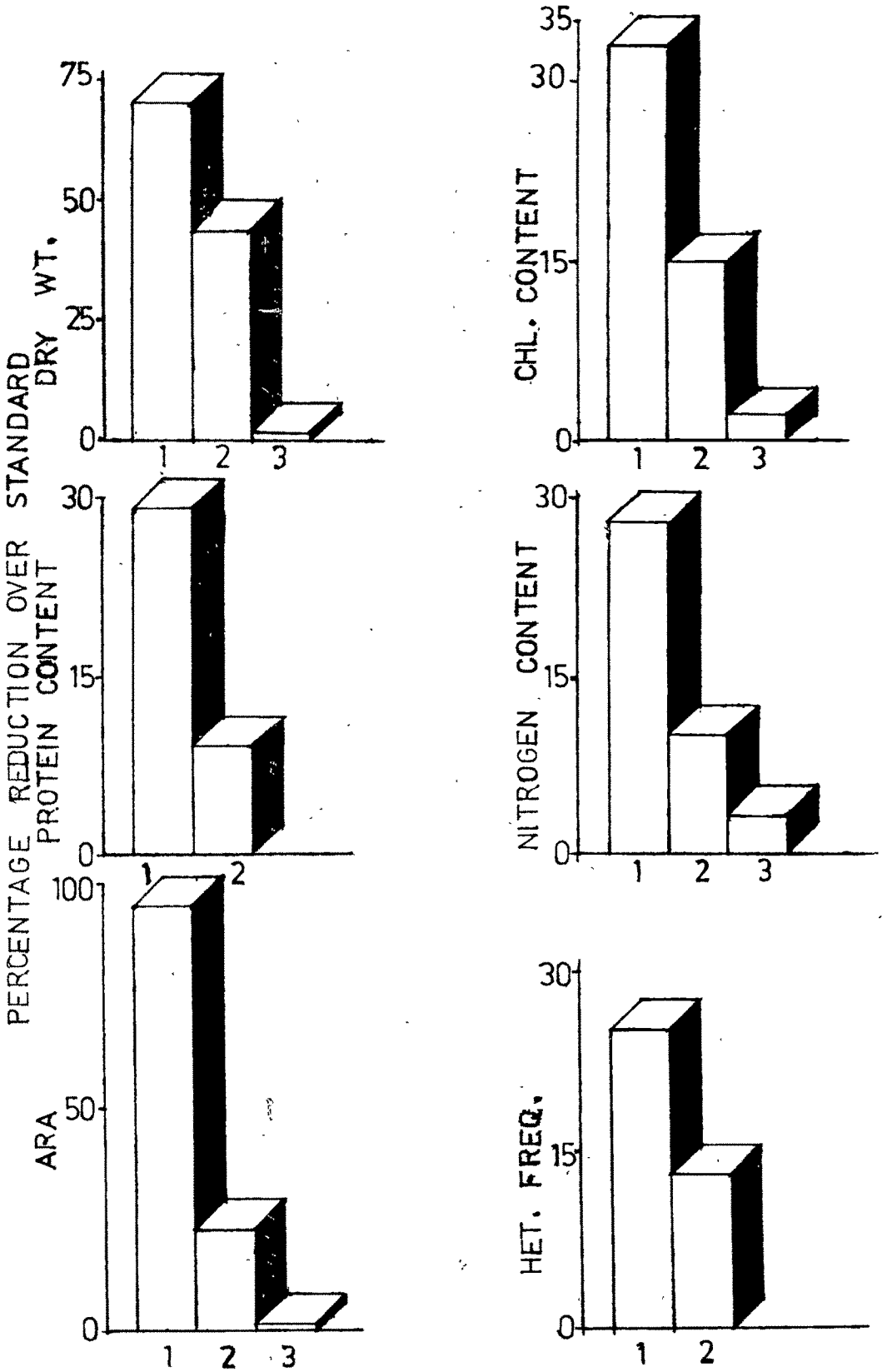
PERCENTAGE REDUCTION OVER STANDARD
DRY WT.
75
50
25
0
1 2 3
PROTEIN CONTENT
30
15
0
1 2
ARA
100
50
0
1 2 3
CHL. CONTENT
35
30
15
0
1 2 3
NITROGEN CONTENT
30
15
0
1 2 3
HET. FREQ.
30
15
0
1 2

Fig. 5

correlation was observed in biomass production of Azolla and acetylene reduction activity (r = 0.888). Similar correlation occurred in nitrogen content accumulated in dry matter (r = 0.962).

Hence 2 ppm of Fe level was found to be the optimal dose for biomass production of Azolla.

Experiment 6

Effect of incorporation of cobalt (Co) at various levels in Watanabe medium on A. pinnata

Watanabe's medium lacks cobalt. To test the effect of cobalt on biomass production and nitrogenase activity of Azolla, the present experiment was conducted. Various levels of Co (0.01, 0.1, 1.0 and 10 ppm) in the form of cobalt chloride, was added into the culture medium. Healthy Azolla plants (300 ± 20 mg) from the axenic stock cultures were transferred to the test media (50 ml). Cultural conditions were maintained as described in Chapter II, Materials and Methods.

Results recorded (Table 12) clearly indicated that addition of cobalt to the culture medium improved the overall biomass production of Azolla. The increase in Co levels from 0.01 to 0.1 ppm showed maximum biomass production of Azolla. The increase was 24% as compared to the growth of

Table 12 : Effect of incorporation of cobalt (Co) in Watanabe medium, on the growth, composition, acetylene reduction activity and heterocyst frequency of A. pinnata

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ±01 mg

Co-levels (ppm)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
Standard	2030	85	0.52	24.8	3.8	656	28
0.01	2270	96	0.52	25.1	3.9	682	29.5
0.1	2470	105	0.53	25.8	4.0	734	30.1
1.0	2170	91	0.52	25.3	3.9	704	30.1
10.0	2030	86	0.52	24.9	3.8	695	29.0
C.D. at 5%	110	5.2	0.09	1.3	0.8	29	1.9

Mean of six replicates

Azolla plants grown without cobalt. Further increase in Co levels (1.0 and 10 ppm) were not favourable for Azolla growth and multiplication. The nitrogenase activity was improved by 12% over the plants cultured in standard medium (Fig. 6).

The effect of cobalt on Azolla composition such as chlorophyll content and nitrogen content was negligible. The nitrogenase activity was improved by incorporation of cobalt (0.1 ppm). Cobalt level at 0.1 ppm gave significantly better results than the rest of the concentrations tested.

Experiment 7

Effect of incorporation of ascorbic acid at various levels in Watanabe medium on growth and nitrogen fixation of A. pinnata

Vitamins are required for the normal growth and development of plants when cultured in synthetic medium. Ascorbic acid at various levels (5, 10, 15 and 20 ppm) was added to Watanabe medium and tested for its effect on biomass production and nitrogenase activity of A. pinnata. Healthy Azolla plants (300 ± 20 mg) were transferred to the test media. Cultural conditions were followed as described in Chapter II, Materials and Methods.

Results showed that there was a significant increase in biomass production of Azolla in presence of ascorbic acid

Fig. 6 Effects of incorporation of Co levels (1=0.01, 2=0.1, 3=1.0 and 4=10.0 ppm) in the Watanabe medium on dry weight, protein content, ARA, chlorophyll content, nitrogen content and heterocyst frequency of *A. pinnata*, after a period of three weeks.

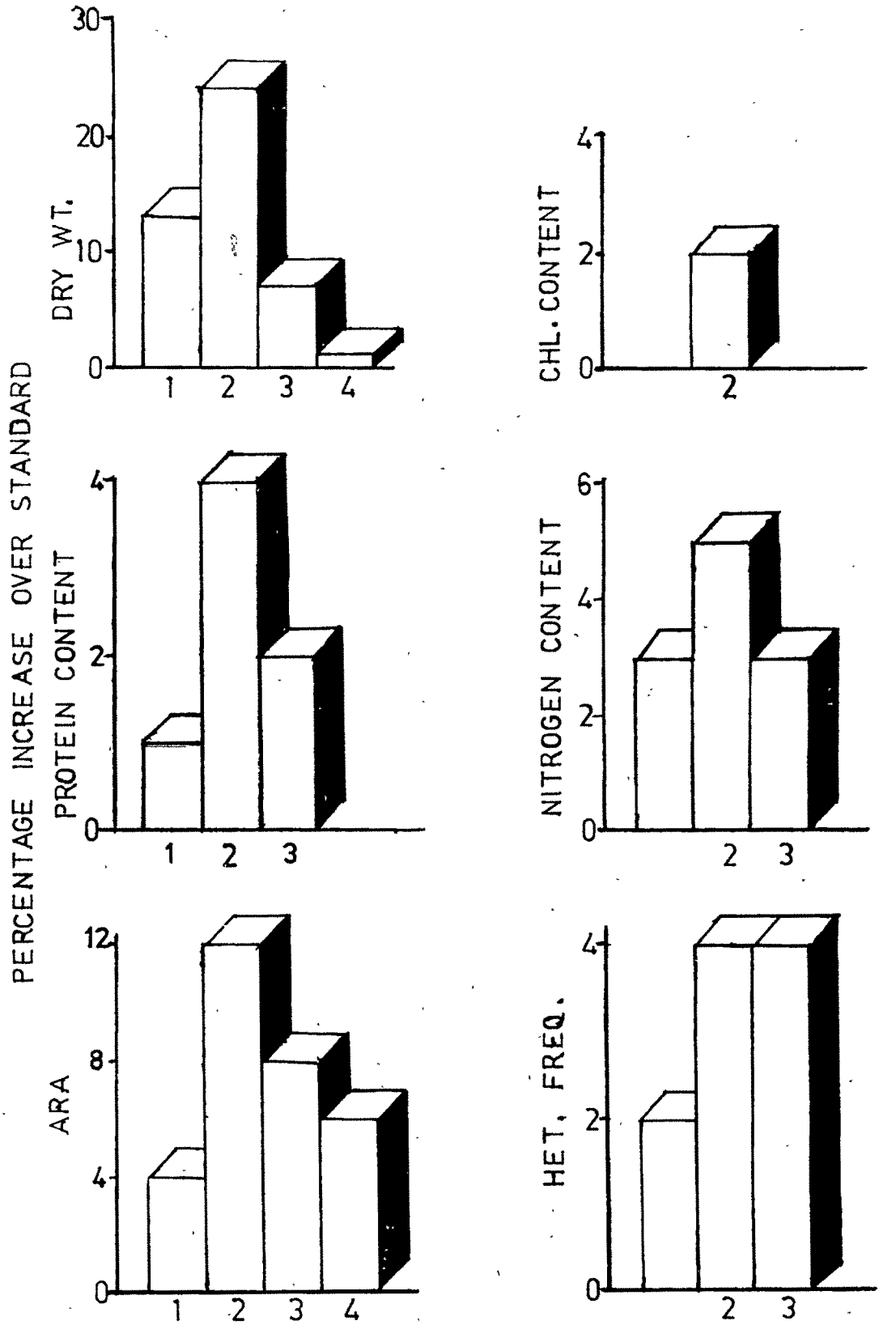
PERCENTAGE INCREASE OVER STANDARD
DRY WT.
30
20
10
0
1 2 3 4
CHL. CONTENT
4
2
0
2
PROTEIN CONTENT
4
2
0
1 2 3
NITROGEN CONTENT
6
4
2
0
2 3
ARA
12
8
4
0
1 2 3 4
HET. FREQ.
4
2
0
2 3

Fig. 6

at all levels tested (Table 13). Highest biomass production was recorded at 10 ppm of ascorbic acid level. But the composition of Azolla viz. chlorophyll contents, protein and nitrogen contents as well as ARA and heterocyst frequency did not significantly change at the levels tested. An increase of 14% in biomass, 2% in chlorophyll content, 5% in nitrogen content, 18% in ARA and 10% in heterocyst frequency was recorded at an ascorbic acid level of 10 ppm in the medium when compared with the corresponding values of the plants grown in standard medium (Fig. 7). A positive correlation existed between biomass produced and ARA ($r = 0.898$).

Experiment 8

Effect of incorporation of combined nitrogen source on A. pinnata

In this experiment, the effects of combined nitrogen added in various forms to the culture medium on biomass production of Azolla and nitrogenase activity were studied. Watanabe's medium was supplemented with combined nitrogen (40 ppm) in the form of ammonium sulphate, ammonium chloride, ammonium nitrate and calcium and potassium nitrates. Ionic balance of the medium was maintained. Healthy Azolla plants (300 ± 20 mg) from axenic stock cultures were transferred to the test medium (50 ml). Cultural conditions were maintained as described in Chapter II, Materials and Methods.

Table 13 : Effect of incorporation of ascorbic acid to Watanabe medium on the growth, composition, acetylene reduction activity and heterocyst frequency of A. pinnata

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ± 01 mg

Ascorbic acid-levels (ppm)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
Control	2630	110	0.53	24.4	3.8	728	31
5	2735	116	0.54	24.8	3.9	771	33
10	2930	125	0.54	24.9	4.0	859	34
15	2790	119	0.53	24.7	3.9	844	33
20	2790	119	0.53	24.7	3.9	841	33
C.D. at 5%	93	4.9	0.07	0.3	0.8	78	2.4

Mean of six replicates

Fig. 7 Effects of ascorbic acid levels (1=5, 2=10, 3=15 and 4=20 ppm) in the Watanabe medium on dry weight, protein content, ARA, chlorophyll content, nitrogen content and heterocyst frequency of A. pinnata, after a period of three weeks.

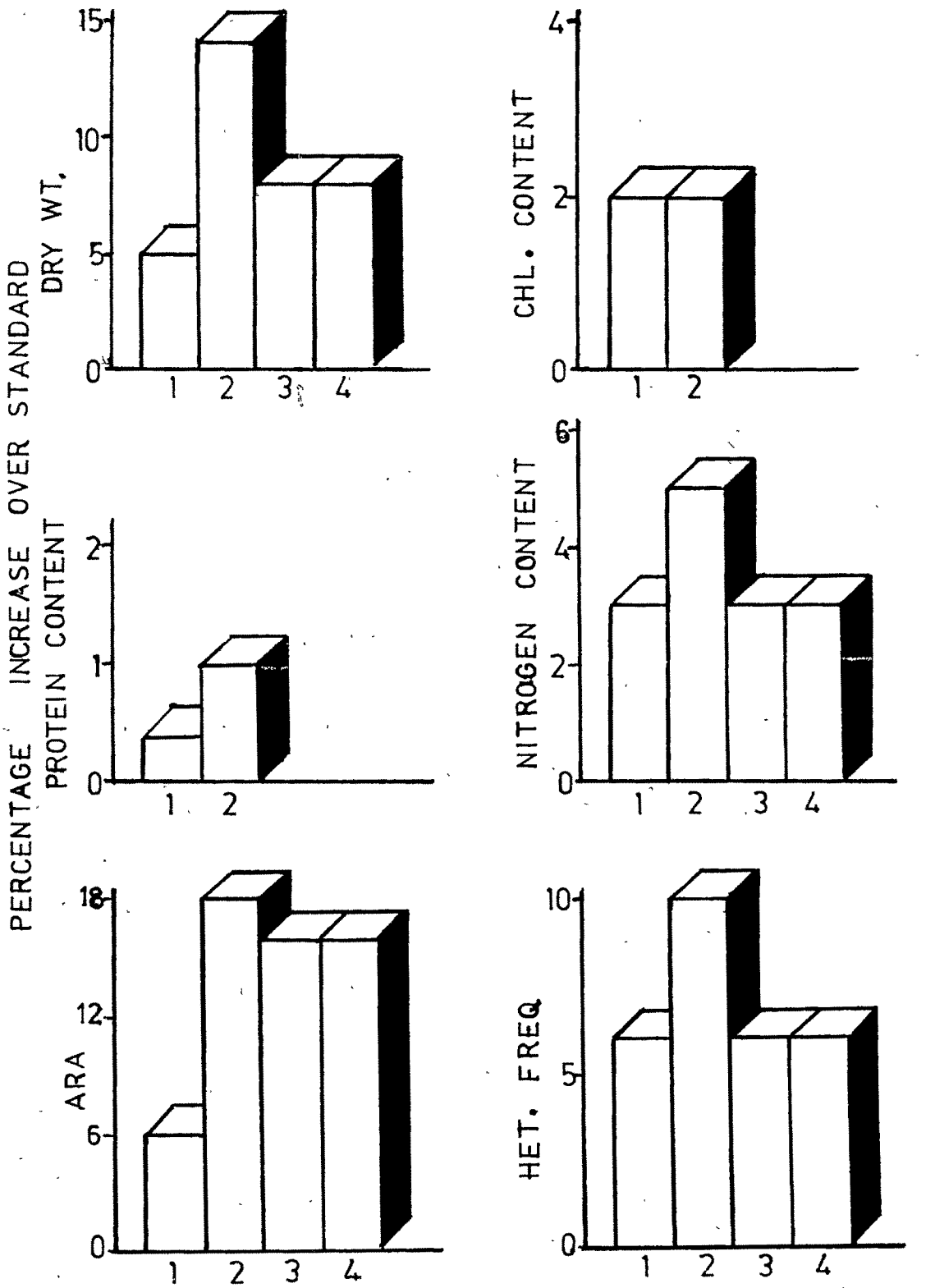
PERCENTAGE INCREASE OVER STANDARD
DRY WT.
15
10
5
0
1 2 3 4
CHL. CONTENT
4
2
0
1 2
PROTEIN CONTENT
2
1
0
1 2
NITROGEN CONTENT
6
4
2
0
1 2 3 4
ARA
18
12
6
0
1 2 3 4
HET. FREQ
10
5
0
1 2 3 4

Fig. 7

After an experimental period of three weeks, the biomass production and the composition of Azolla remained unaffected (Table 14), while the heterocyst frequency was reduced to 21% bringing a corresponding reduction in nitrogenase activity to 59% in presence of ammonium sulphate as compared to Azolla plants cultured in nitrogen free medium (Fig. 8). The percentage reductions in the frequency of heterocyst and activity of nitrogenase were more in the presence of ammonium salts than in the presence of calcium and potassium nitrates. The nitrogen contents of the dry matter remained unaffected.

Thus it was evident that presence of nitrogen source in the culture media suppressed heterocyst differentiation of Anabaena azollae with corresponding decrease in nitrogenase activity of Azolla. Irrespective of any source of nitrogen, biomass, chlorophyll, protein and nitrogen contents were at par with the standard Watanabe medium, however acetylene reduction activity and heterocyst frequency were significantly below those recorded using the standard Watanabe medium.

Experiment 9

Comparison of the growth and nitrogenase activity of A. pinnata grown in standard Watanabe medium and modified medium, considering the current studies

The present experiment was conducted to find out the

Table 14 : Effect of various combined nitrogen sources on the growth, composition, acetylene reduction activity and heterocyst frequency of *A. pinnata*

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ± 01 mg

Nitrogen source (40 ppm-N)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
Standard	2070	87	0.52	24.6	3.9	688	28
$(NH_4)_2SO_4$	2060	86	0.52	24.5	3.8	284	22
NH_4Cl	2050	85	0.52	24.5	3.9	261	21
NH_4NO_3	2020	84	0.52	24.5	3.9	312	25
$Ca(NO_3)_2$ + KNO_3	2070	85	0.53	24.6	3.9	430	27
C.D. at 5%	68	6	0.09	1.8	0.7	49	1.3

Mean of six replicates

Fig. 8 Effects of combined nitrogen (40 ppm of N levels in the form of 1 = Ammonium sulphate, 2 = Ammonium chloride, 3 = Ammonium nitrate and 4 = potassium nitrate + calcium nitrate) added in Watanabe medium on ARA and heterocyst frequency of *A. pinnata*, after a period of three weeks.

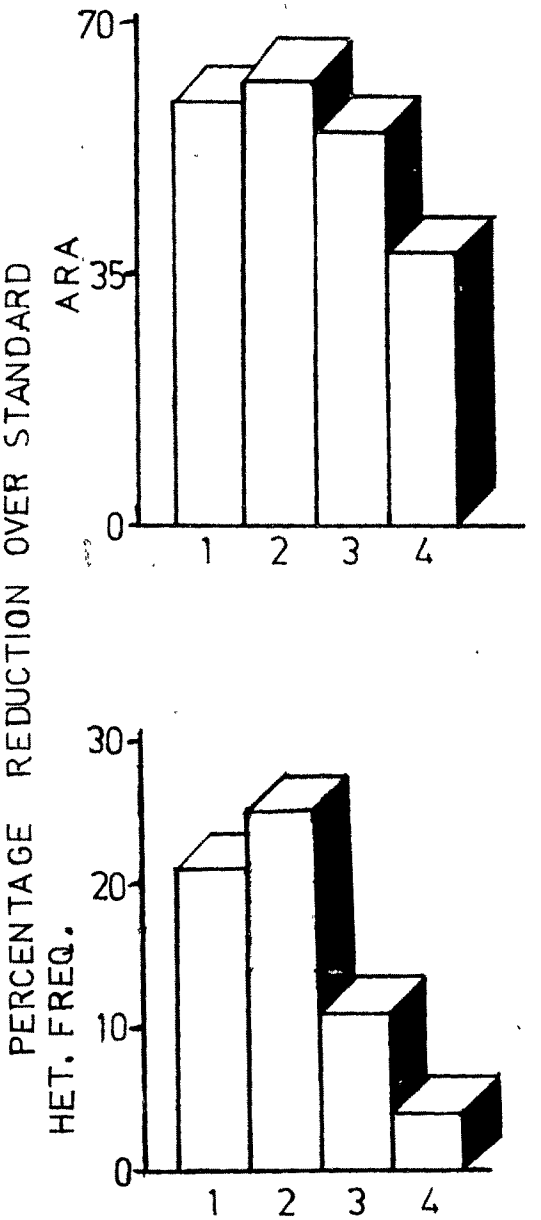
PERCENTAGE REDUCTION OVER STANDARD
ARA
70
35
0
1
2
3
4
HET. FREQ.
30
20
10
0
1
2
3
4

Fig. 8

effect of a modified Watanabe's medium (30 ppm of P and 0.1 ppm of Co) on production of biomass and its nitrogenase activity of Azolla as compared with plants grown in standard Watanabe medium. Healthy Azolla plants (300 ± 20 mg) from stock cultures were transferred to the Watanabe medium (standard) and to the modified medium. Culture flasks were incubated as described in the Chapter II, Materials and Methods. Chlorophyll, protein and nitrogen contents, ARA and heterocyst frequency were determined according to the methods described in Chapter II, Materials and Methods.

Results presented in Table 15 clearly indicated that modified medium supported a 2-fold increase in biomass production over that grown in standard Watanabe medium. Statistical analysis of the data, showed that the increase of the biomass production was significant at 1% probability level. The biomass production showed 30% increase in terms of fresh and dry weight when compared to the corresponding values of the plants grown in standard Watanabe medium. Besides biomass production, the heterocyst frequency showed 14% increase with corresponding 24% increase in ARA.

Experiment 10

Studies with respects to preservation of Azolla

In all the experiments described in this work, Azolla has been cultured in a liquid medium. It was of interest to

Table 15 : Comparison of growth, composition and nitrogenase activity of *A. pinnata* grown in Watanabe medium (standard) and modified medium

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ± 01 mg

Medium	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
Watanabe medium (standard)	2045±72	86±4	0.53±0.08	24.7±1.2	3.8±0.8	672±84	28±2.4
Modified medium	2675±68	114±9	0.54±0.06	26.8±3.4	4.1±0.6	832±78	32±3.2

Mean of six replicates

study the culture of Azolla on a semi-solid medium. Azolla plants (300 ± 20 mg) were transferred to petri-dishes containing Watanabe medium (15 ml) solidified with 0.9% agar-agar. The petri-dishes were sealed and the cultures were incubated in the culture room at 25 ± 2°C with 16 hours photoperiod (1000 Lux).

The growth of Azolla on semi-solid medium was extremely slow. Only 2.5-fold increase in biomass production was recorded after a culture period of three weeks (Table 16). But it was observed that, without subculturing, the plants survived for three months. Therefore, although there was not much increase in biomass production, subculturing on semi-solid medium was advantageous for preserving and transporting of Azolla plants.

SECTION B : Hormonal studies

Application of phytohormones to plants grown in sterile nutrient media enhances their growth and development.

Experiment 11

Effect of phytohormones on biomass production and nitrogenase activity of A. pinnata

Phytohormones such as kinetin (Kn), idole-3-acetic acid (IAA), 2, 4-dichlorophenoxy acetic acid (2, 4-D) and

Table 16 : Biomass production of Azolle cultured on semi-solid Watanabe medium

Inoculum : Fresh wt. = 300 ± 20 mg
Dry wt. = 13 ± 01 mg

Period (weeks)	Fresh weight (mg)	Dry weight (mg)
1	398 ± 27	17 ± 2.3
2	518 ± 32	24 ± 8.7
3	780 ± 48	38 ± 8.6

Mean of six replicates with S.D.

gibberellic acid (GA_3) at various levels were incorporated in the Watanabe medium. Healthy Azolla plants (300 ± 20 mg) were transferred to each of these media. Cultural conditions were maintained as described in the Chapter II, Materials and Methods.

a) Kinetin (Kn)

Incorporation of Kinetin at various concentrations (0.01, 0.1, 1.0 and 10.0 ppm) to Watanabe medium did not affect the growth and nitrogen fixation of A. pinnata during three weeks of experimental period (Table 17). No significant changes were observed in the biomass production, chlorophyll content, protein content, acetylene reduction activity at the Kn levels tested. No exogenous supply of cytokinin in the form of kinetin was found necessary for Azolla culture.

b) Indole 3-acetic acid (IAA)

It was noticed that after three weeks the biomass production of Azolla and nitrogenase activity did not increase by incorporating IAA to the culture medium (Table 18). The various concentrations 0.01 ppm to 10 ppm were ineffective in increasing Azolla biomass. The composition of Azolla viz. chlorophyll, protein and nitrogen contents, also remained unchanged.

Table 17 : Effect of kinetin (Kn) on the growth, composition, acetylene reduction activity and heterocyst frequency of *A. pinneta*

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ± 01 mg

Kinetin levels (ppm)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
Standard	2680	114	0.53	24.9	3.9	739	31
0.01	2690	114	0.53	24.9	3.9	741	31
0.1	2710	116	0.53	25.0	3.9	749	31
1.0	2690	114	0.53	24.7	3.9	747	31
10.0	2670	113	0.53	24.7	3.9	733	31
C.D.at 5%	52	6.3	0.1	1.2	0.6	49	0.7

Mean of six replicates

Table 18 : Effect of indole-3-acetic acid (IAA) on the growth, composition, acetylene reduction activity and heterocyst frequency of *A. pinnata*

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ± 01 mg

IAA-levels (ppm)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
Standard	2690	114	0.53	24.8	3.9	728	31
0.01	2710	117	0.53	24.9	3.9	732	31
0.1	2710	118	0.53	24.8	3.9	744	31
1.0	2700	116	0.53	24.8	3.9	724	31
10.0	2660	112	0.53	24.7	3.8	727	30
C.D. at 5%	72	9.2	0.09	1.7	1.3	56	1.3

Mean of six replicates

Hence, it was evident that application of IAA was not necessary for Azolla biomass production.

c) 2, 4-Dichlorophenoxyacetic acid (2, 4-D)

Azolla cultured in media containing various concentrations of 2, 4-D (0.01, 0.1, 1.0 and 10 ppm) showed that upto 0.1 ppm concentrations, the biomass production and ARA remained unaffected (Table 19). Increase in 2, 4-D concentration to 1 ppm, resulted in reduction of biomass by 12% and ARA by 13% respectively. Chlorophyll content and nitrogen contents were reduced by 6% and 3% respectively. Further increase in 2, 4-D concentration to 10 ppm resulted in death of Azolla plants within a short period.

d) Gibberellic acid (GA_3)

Gibberellic acid was incorporated into Watanabe culture medium at various concentrations (0.01, 0.1, 1.0 and 10.0 ppm). At low levels of GA_3 (0.01 and 0.1 ppm) biomass production of Azolla showed significant increase when compared with Azolla cultured in media without it (Table 20). The increase observed was 10% in biomass production and 5% in ARA as compared with those values of the plants cultured in standard medium (Fig. 9). A positive correlation existed between biomass and ARA of Azolla ($r = 0.990$) and dry matter of Azolla with nitrogen contents ($r = 0.752$).

Table 19 : Effect of 2, 4-dichlorophenoxy acetic acid (2, 4-D) on the growth, composition, acetylene reduction activity and heterocyst frequency of *A. pinnata*

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ± 01 mg

2, 4-D-levels (ppm)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
Standard	2680	112	0.53	24.9	3.9	738	31
0.01	2680	111	0.53	24.9	3.9	740	31
0.1	2670	110	0.52	24.6	3.9	729	31
1.0	2350	99	0.50	23.9	3.8	640	30
10.0	-	-	-	-	-	-	-
C.D. at 5%	138	7.8	0.02	1.6	1.3	64	1.8

Mean of six replicates

Table 20 : Effect of gibberellic acid (GA_3) on the growth, composition, acetylene reduction activity and heterocyst frequency of A. pinnata

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ± 01 mg

GA_3 levels (ppm)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
Standard	2650	110	0.53	24.7	3.8	732	31
0.01	2710	115	0.53	24.7	3.8	748	31
0.1	2840	121	0.54	24.9	3.9	769	32
1.0	2760	117	0.53	24.8	3.9	753	31
10.0	2740	116	0.53	24.7	3.8	732	31
C.D. at 5%	89	5.2	0.08	1.2	0.2	48	1.7

Mean of six replicates

Fig. 9 Effects of incorporation of gibberellic acid at various levels (1=0.01, 2=0.1, 3=1.0 and 4=10.0 ppm) in the Watanabe medium on dry weight, protein content, ARA, chlorophyll content, nitrogen content and heterocyst frequency of A. pinnata, after a period of three weeks.

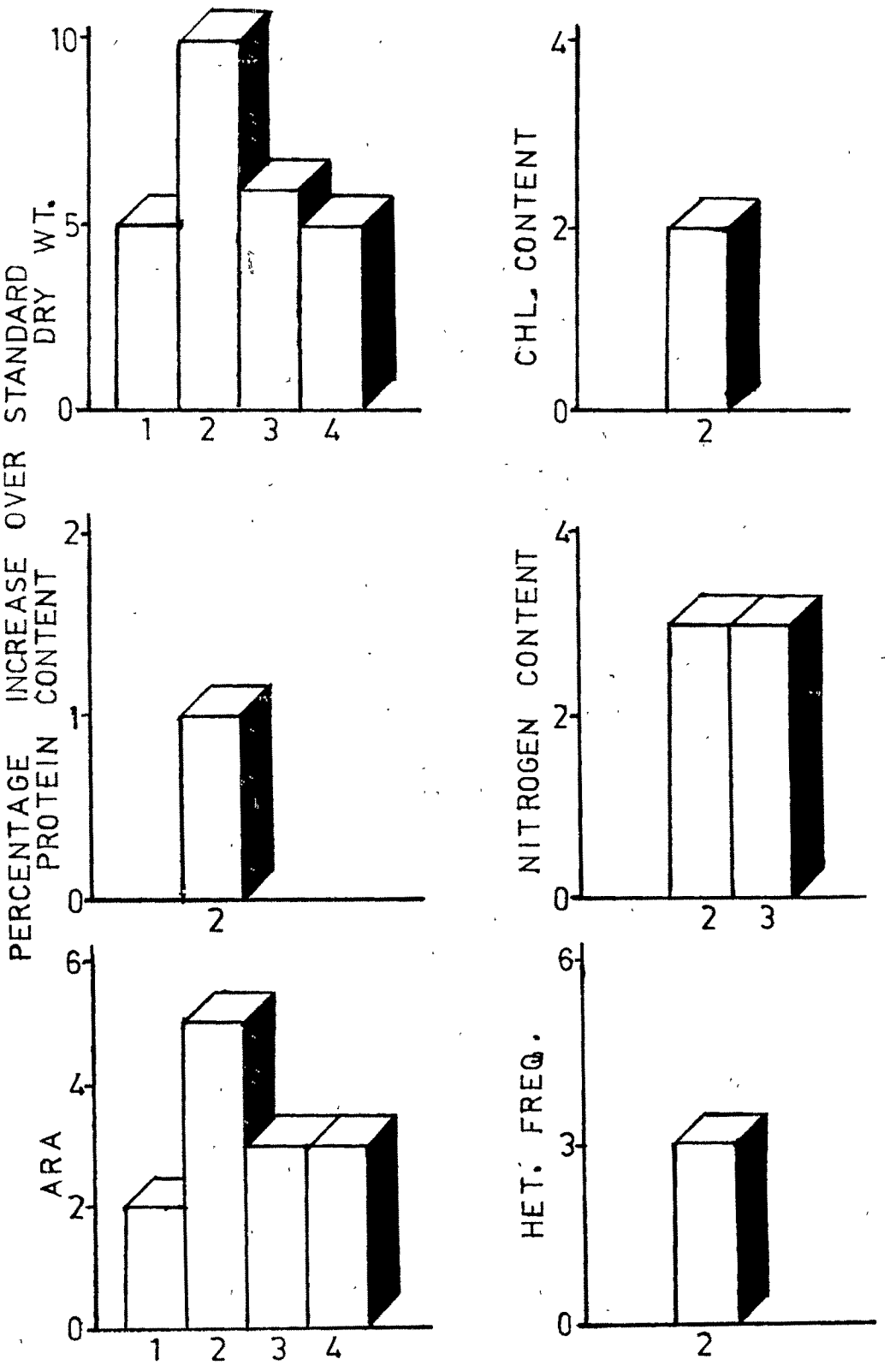
PERCENTAGE INCREASE OVER STANDARD
DRY WT.
10
5
0
1 2 3 4
CHL. CONTENT
4
2
0
2
PROTEIN CONTENT
2
1
0
2
NITROGEN CONTENT
4
2
0
2 3
ARA
6
4
2
0
1 2 3 4
HET. FREQ.
6
3
0
2

Fig. 9

SECTION C: Anatomical studies

Experiment 12

Anatomical studies on A. pinnata

Azolla plants grown in vitro were used to study anatomical details of the complex. To study the anatomical details, procedures described in the Chapter II, Materials and Methods (11) have been followed.

Each leaf of Azolla was bilobed, the ventral lobe nearly achlorophyllous and increased in thickness from one cell at their distal end to two or more cells at the proximal end. The dorsal chlorophyllous lobe contained the algal symbiont Anabaena azollae in an internal cavity (Plate 8,a) The leaf cavity was lined by two types of epidermal outgrowths in the form of hairs (Plate 8,b). Branched hairs were only two, while simple unbranched hairs showed definite number from first leaf to fifteenth leaf in its serial order of development. The branched hairs consisted three to four cells and they were located in similar positions in every leaf cavity, always on the path of the foliar trace (Plate 8,c). The highest number of simple hairs recorded was 22 in the fifteenth leaf and it remained constant upto twentieth leaf (Plate 8,d). In each of the dorsal leaf cavity Anabaena trichomes present consisted of vegetative cells and a few heterocysts (Plate 8,e). Heterocysts were

Plate 8 Photomicrograph of dorsal leaf lobe/s of *Azolla pinnata* showing

a) leaf lobes with Anabaena azollae (600 x)

b) branched hair (BH) folier trace (900 x)

c) magnified view of branched hair (BH) (1200 x)

d) unbranched hair (UB) (900 x)

e) isolated *Anabaena azollae* (1000 x)

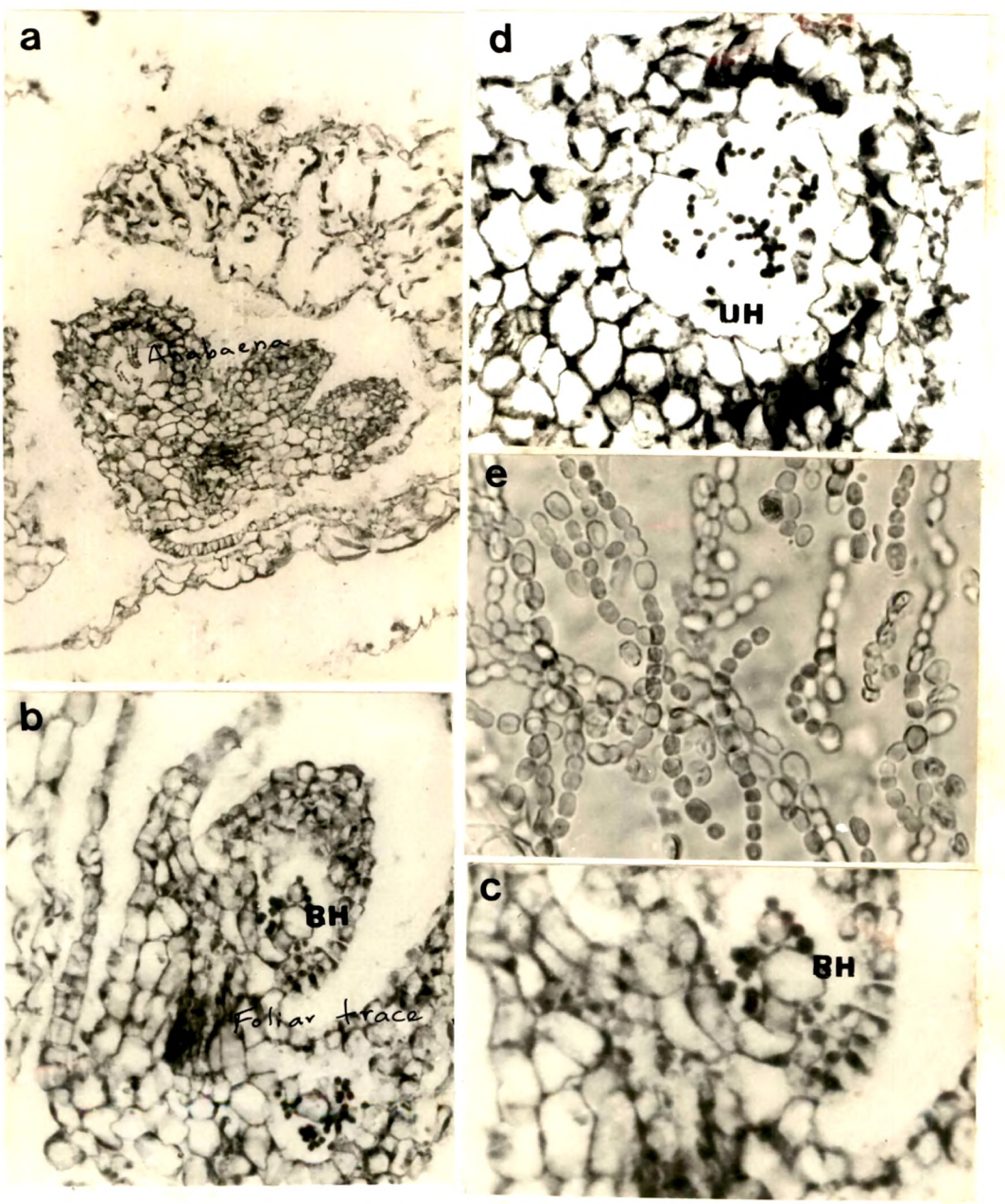

Plate 8

observed from Anabaena isolated from third leaf onwards, reached a maximum in the fifteenth leaf (Fig. 10). A direct correlation between the heterocyst frequency in Anabaena trichomes and the number of simple hairs produced was noted. A few Anabaena cells were present around the shoot apex of Azolla. Studies on Anabaena isolated from fresh material by fluorescence microscopy showed that chlorophyll and phycocyanin pigments were present in vegetative cells and heterocysts (Plate 9, a and b).

SECTION D : Salinity studies

The studies on the salinity, induced by the addition of sodium chloride to the Watanabe's medium were conducted to examine its effects on the growth, composition and nitrogen fixation of A. pinnata under aseptic conditions.

Experiment 13

Effect of sodium chloride-induced salinity on A. pinnata

Healthy Azolla plants (300 ± 20 mg) from stock cultures were transferred to Watanabe medium (50 ml) supplemented with various levels of sodium chloride (5, 10, 20, 30, 40 and 50 mM). Cultural conditions and procedures for analysis were followed as described in Chapter II, Materials and Methods.

Fig. 10 Correlation between percent heterocyst frequency of isolated Anabaena azollae and Azolla leaves in their serial order of development.

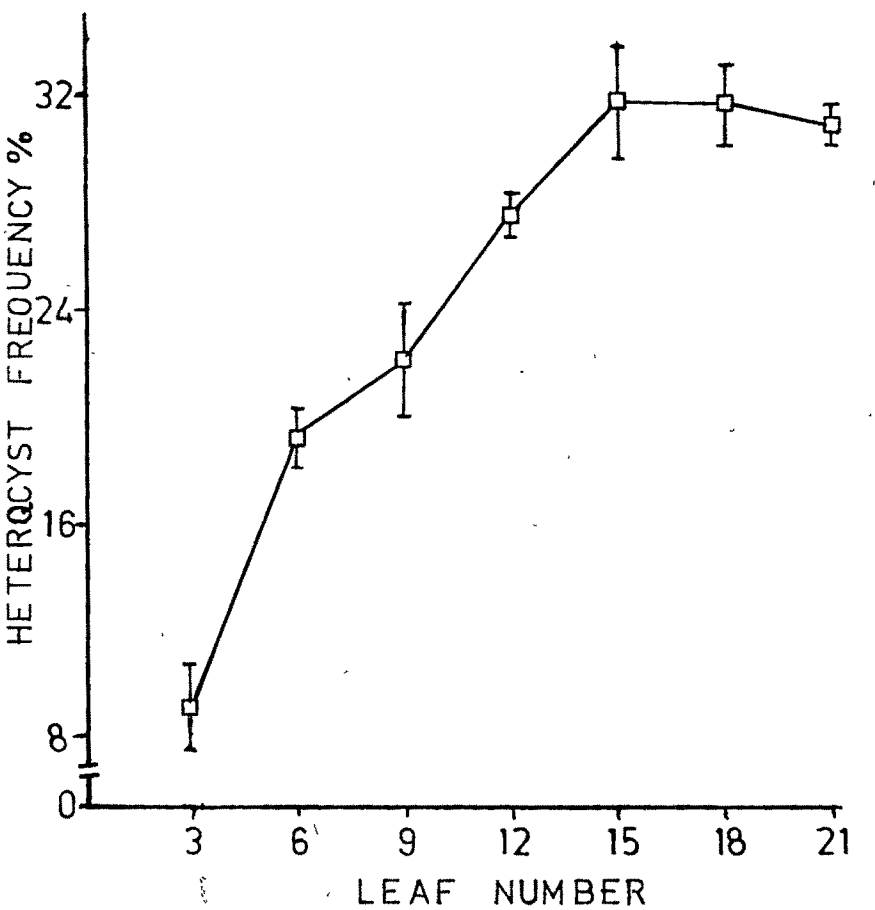

Fig. 10

Plate 9 a) Fluorescence of chlorophyll in vegetative cells and heterocysts in isolated Anabaena azollae (1000 x)

b) Fluorescence of phycocyanin in vegetative cells and heterocysts in isolated Anabaena azollae (1000 x)

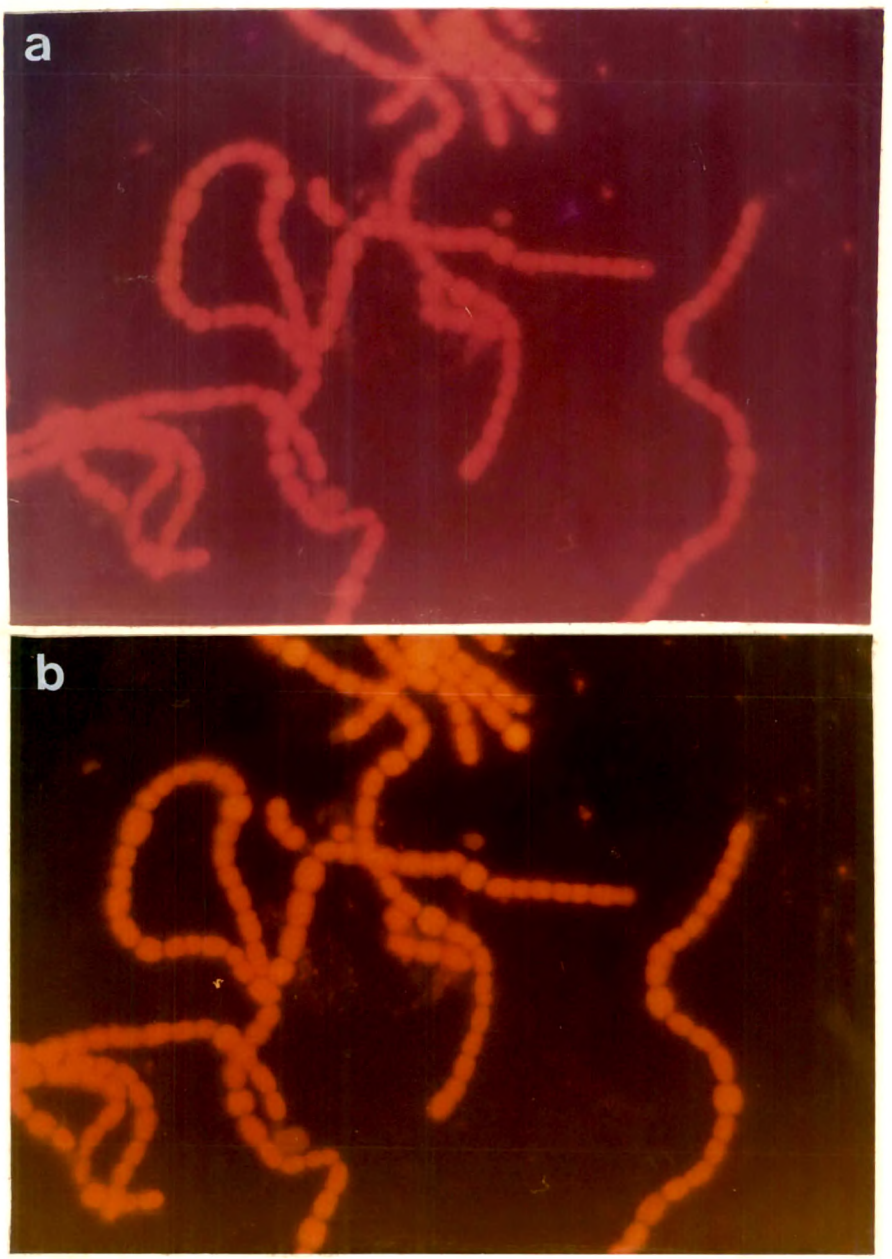

Plate 9

a) Effect on biomass

Azolla plants subjected to low levels of salinity (5 and 10 mM) were similar to the plants grown in standard culture medium. There was no significant reduction in biomass produced. With the increase in salinity to 20 mM of sodium chloride, Azolla plants became pale green and roots were short and dark brown (Plate 10). Azolla cultured at 30 and 40 mM of sodium chloride levels, leaves showed chlorosis and plants were devoid of roots. Biomass production was reduced by 30% and 72% at 20 mM and 40 mM of NaCl levels respectively, when compared with the biomass produced by Azolla cultured in standard medium (Table 21).

b) Effect on composition

The growth parameters viz. chlorophyll contents, protein and nitrogen contents also showed progressive reduction as the levels of sodium chloride increased in the culture medium. At 20 mM of NaCl level, 23%, 16% and 3% reduction in chlorophyll, protein and nitrogen contents respectively were observed when compared to plants cultured in standard medium. At 40 mM, 26% reduction in nitrogen content was noted.

c) Effect on nitrogenase activity

The heterocyst frequency in the symbiont Anabaena isolated from Azolla treated with 40 mM of sodium chloride

Plate 10 Effects of sodium chloride levels (a = control, b=5, c=10, d=20, e=30 and f=40 mM) on *A. pinnata*

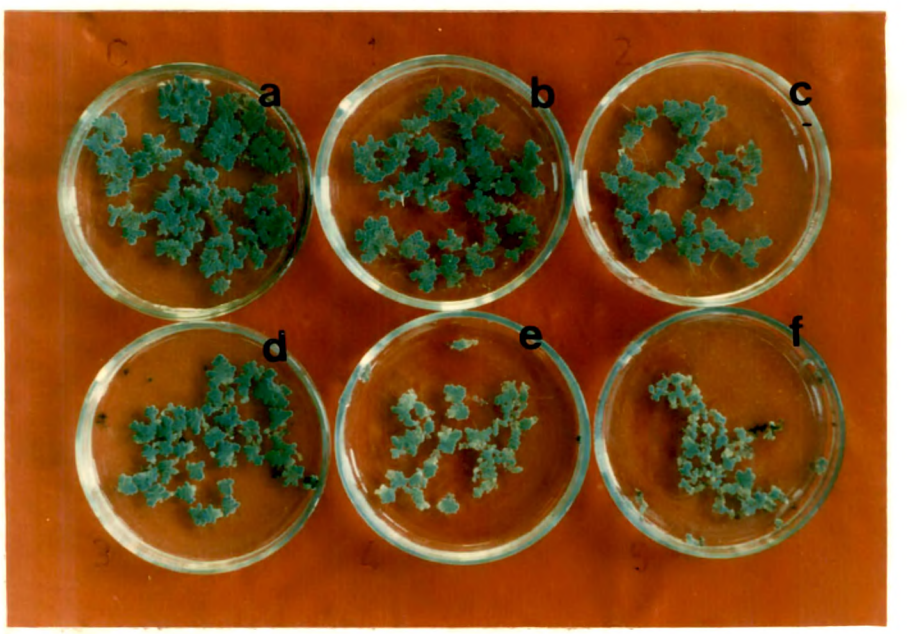

Plate 10

Table 21 : Effect of sodium chloride-induced salinity on the growth, composition, acetylene reduction activity and heterocyst frequency of *A. pinnata*

Inoculum : Fresh wt. = 300 ± 20 mg Dry wt. = 13 ± 01 mg

Sodium chloride-levels (mM)	Fresh weight (mg)	Dry weight (mg)	Total chlorophyll content (mg/g fr.wt)	Protein content (mg/g fr.wt.)	Nitrogen content (% by dry wt. basis)	ARA (n mole C_2H_4 formed/g fr. wt./hr.)	Heterocyst frequency (%)
Standard	2590	103	0.53	28.3	3.9	685	32.7
5	2570	102	0.53	28.2	3.9	680	32.6
10	2530	101	0.52	28.0	3.9	668	32.0
20	1760	72	0.41	23.9	3.8	530	28.6
30	1080	39	0.31	18.6	3.1	411	26.5
40	720	29	0.28	17.8	2.9	213	23.8
C.D. at 5%	134	7.3	0.11	2.6	1.9	76	1.5

Mean of six replicates

recorded 69% reduction in acetylene reduction activity and 27% reduction in heterocyst frequency (Fig. 11).

Positive correlations between biomass production of Azolla and acetylene reduction activity (r = 0.978) as well as with nitrogen content accumulated in dry matter (r=0.983) were observed.

Thus it was evident that A. pinnata could tolerate 10 mM of sodium chloride present in the medium.

d) Effect on ammonia assimilating enzymes

Three principal ammonia assimilating enzymes, glutamine synthetase (GS), glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) were extracted from sodium chloride treated Azolla. The extraction and estimation procedures followed for these enzymes were as described in Chapter II, Materials and Methods (10b-i, ii and iii).

Results obtained showed that at low level of salinity (5 mM), the GS activity was reduced by 6% while GDH and GOGAT remained unaffected (Fig. 12). Progressive reduction in GS activity of Azolla was recorded as the treatment levels of sodium chloride increased. At 20 mM of sodium chloride in the medium GS activity was reduced by 39% while at 40 mM it was reduced by 77% as compared with GS activity exhibited by

Fig. 11 Effects of sodium chloride levels (1=5, 2=10, 3=20, 4=30 and 5=40 mM) incorporated in Watanabe medium on dry weight, protein content, ARA, chlorophyll content, nitrogen content and heterocyst frequency of *A. pinnata*, after a period of three weeks.

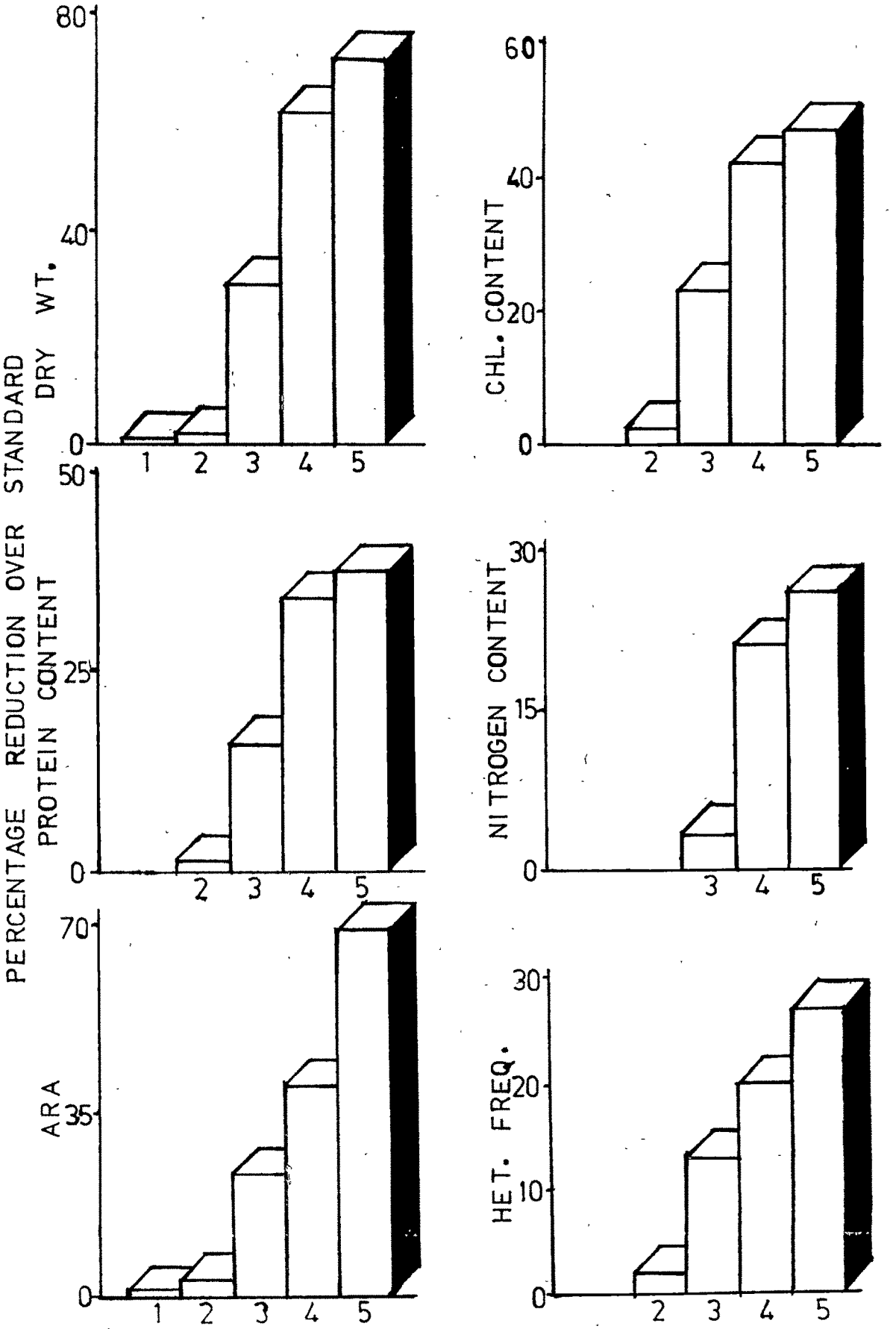
PERCENTAGE REDUCTION OVER STANDARD
DRY WT.
80
40
0
1 2 3 4 5
CHL. CONTENT
60
40
20
0
2 3 4 5
PROTEIN CONTENT
50
25
0
2 3 4 5
NITROGEN CONTENT
30
15
0
3 4 5
ARA
70
35
0
1 2 3 4 5
HET. FREQ.
30
20
10
0
2 3 4 5

Fig. 11

Fig. 12 Effects of sodium chloride levels (C = control, 1=5, 2=10, 3=20, 4=30 and 5=40 mM) on ammonia assimilating enzymes: Glutamine synthetase (GS) Glutamate dehydrogenase (GDH) and Glutamate synthase (GOGAT).

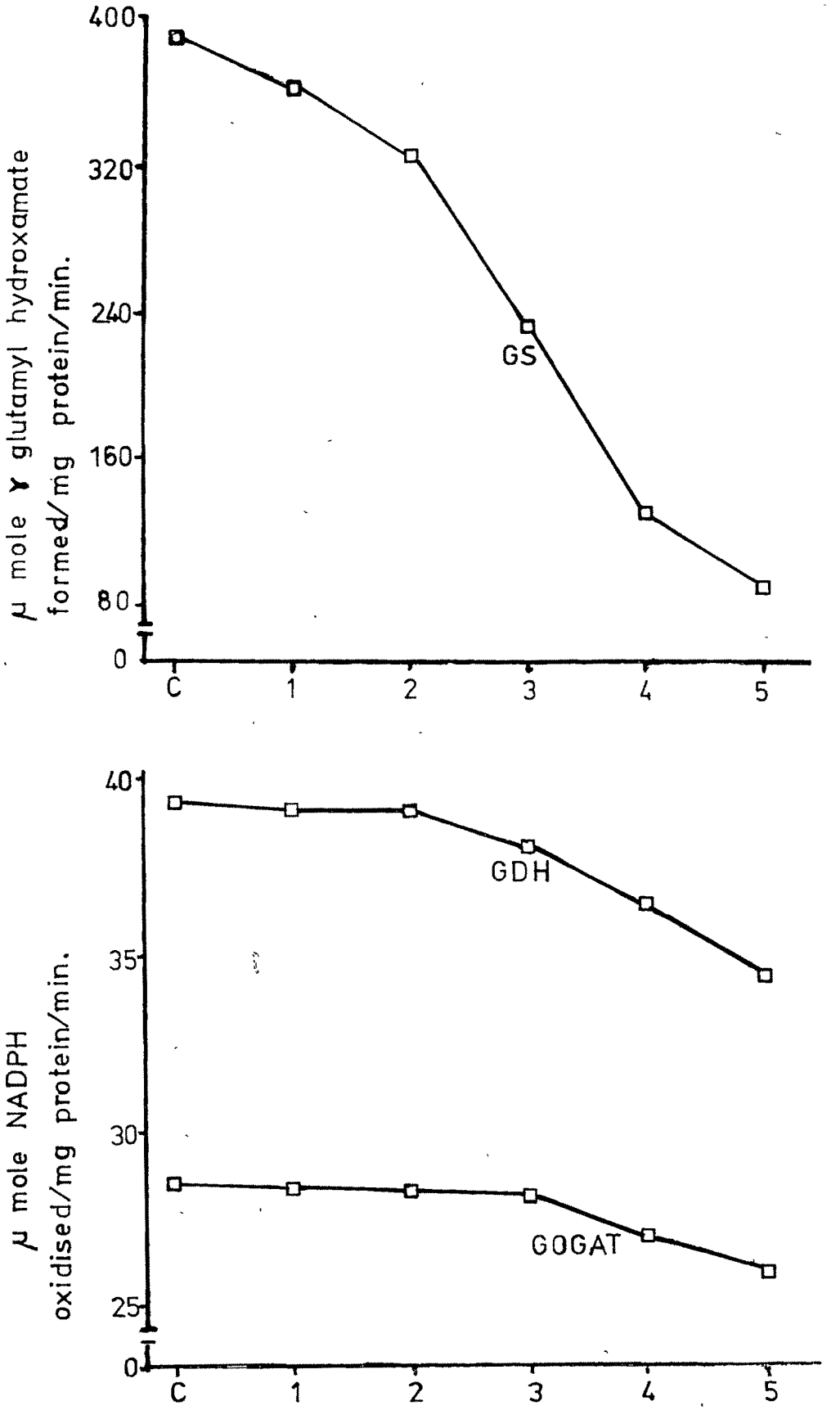
μ mole γ glutamyl hydroxamate formed/mg protein/min.
400
320
240
160
80
0
GS
C
1
2
3
4
5
μ mole NADPH oxidised/mg protein/min.
40
35
30
25
0
GDH
GOGAT
C
1
2
3
4
5

Fig. 12

Azolla plants cultured in standard medium. The GDH and GOGAT activities of Azolla were not much affected due to the presence of various concentrations of sodium chloride in the culture media. The maximum reduction recorded at the highest concentration of sodium chloride treated (40 mM) Azolla was 8% and 13% in GDH and GOGAT respectively, when compared with these activities of Azolla grown in standard medium.

e) Effect on the ionic constituents of Azolla

Ionic constituents from Azolla, cultured in media supplemented with various concentration of sodium chloride were analysed according to the procedures described in Chapter II, Materials and Methods (9).

Sodium and chloride ions in Azolla showed gradual increase corresponding to the increase in sodium chloride levels of the medium. The sodium content increased by 77% while chloride content increased by 151% at 40 mM of sodium chloride concentration present in culture medium when compared with these contents present in Azolla grown in standard medium.

Potassium content was severely affected when compared with calcium, phosphorus and magnesium contents due to salinity. At lower concentrations of sodium chloride in the

medium (5 and 10 mM) a slight reduction occurred in potassium content. With increasing levels of sodium chloride levels viz. 20, 30 and 40 mM in medium, the reduction by 10%, 34% and 62% in potassium contents occurred respectively when compared with Azolla plants cultured in standard medium.

At 10 mM of sodium chloride level 4% reduction in calcium content was recorded. Further increase in sodium chloride levels in culture medium upto 40 mM reduced calcium content by 41% .

Phosphorous and magnesium contents of Azolla were not affected upto 10 mM of sodium chloride level present in culture medium. Phosphorous and magnesium contents were adversely affected at higher levels of sodium chloride present in the culture media (Table 22).

Azolla pinnata can tolerate sodium chloride upto 10 mM level without decreasing its biomass production. Beyond this level upto 40 mM, A. pinnata though decreases the biomass production level, however grew reasonably well. It was possible that these plants helped in gradual removal of sodium chloride levels from saline water.

f) Effect on symbiont Anabaena azollae

Anabaena, isolated from the fifteenth leaf of Azolla

Table 22 : Ionic composition of *A. pinnata* grown in media containing various levels of sodium chloride

NaCl levels (mM)	Sodium	Chloride	Calcium	Potassium	Phosphorous	Magnesium
			(mg/g dry weight)			
Standard	4.3	7.9	8.9	29.8	9.3	4.9
5	4.9	8.6	8.8	29.3	9.3	4.9
10	5.1	9.1	8.5	29.0	9.1	4.9
20	5.6	10.7	7.3	26.1	9.0	4.9
30	6.3	15.3	6.1	19.8	8.9	4.7
40	7.6	19.8	5.3	11.4	8.6	4.5

Mean of three replicates

treated to various concentrations of sodium chloride was studied for the pigmentation changes in the vegetative cells and heterocysts. The chlorophyll contents and phycocyanin contents from both these types of cells were estimated by their fluorescence as described in Chapter II, Materials and Methods (10 a).

An increase in the phycocyanin contents by 2% and 3% in vegetative cells and heterocysts respectively were observed in Anabaena isolated from Azolla treated with 5 mM sodium chloride (Fig. 13). But at this concentration of sodium chloride no change in chlorophyll contents in both the types of cells was noted. With further increase in sodium chloride in culture medium of Azolla, corresponding, decrease in chlorophyll as well as phycocyanin contents occurred in both the types of cells (Plate 11, a, b; Plate 12, a, b, c, d). Chlorophyll pigment completely disappeared in both the types of cells of Anabaena treated with 40 mM of sodium chloride. The phycocyanin content was reduced by 73% and 61% in vegetative cell and heterocyst respectively. Hence, it was evident that salinity affected the pattern of pigmentation of the symbiont.

Fig. 13 Changes in chlorophyll and phycocyanin contents in Anabaena azollae isolated from various levels of sodium chloride (1=5, 2=10, 3=20, 4=30 and 5=40 mM) treated A. pinnata.

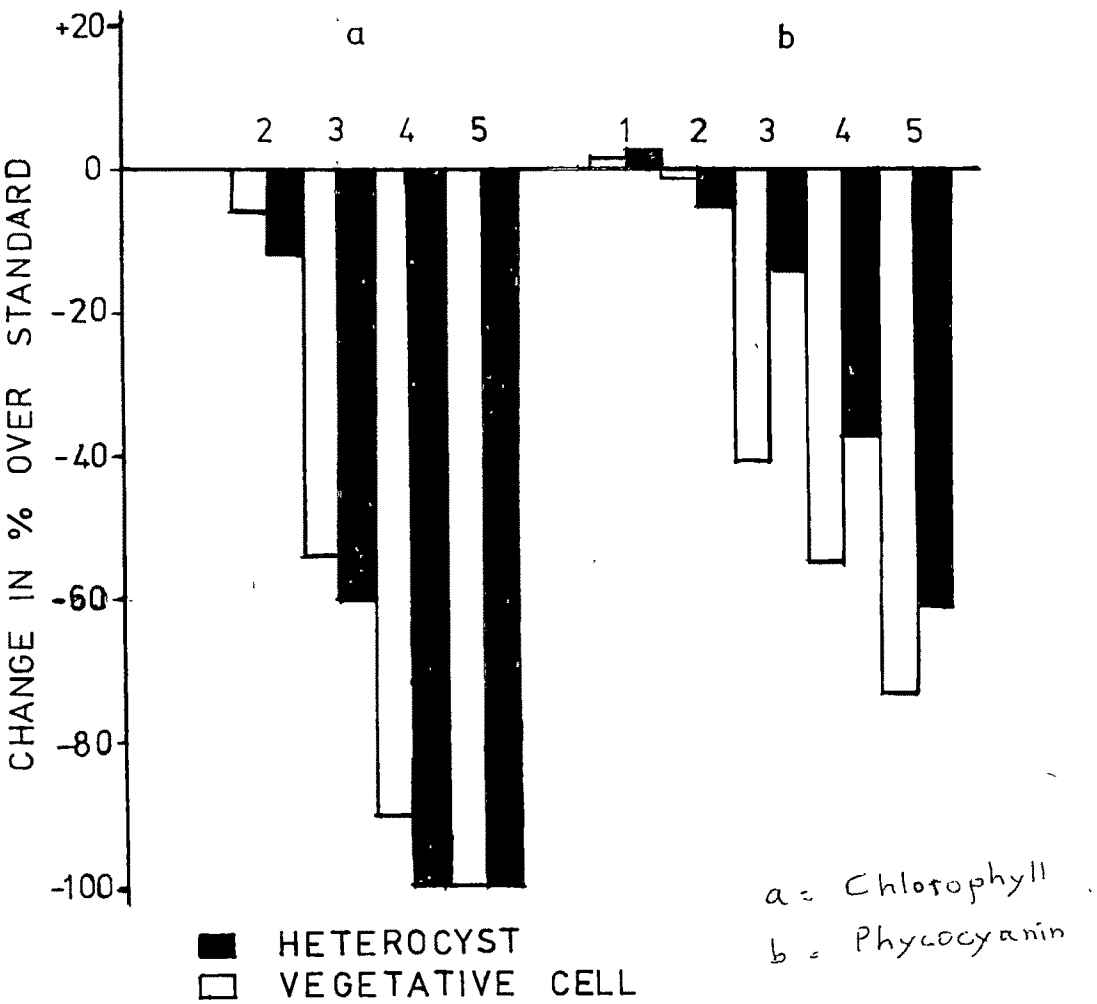
+20
0
-20
-40
-60
-80
-100
CHANGE IN % OVER STANDARD
a
b
2 3 4 5
1 2 3 4 5
HETEROCYST
VEGETATIVE CELL
a = Chlorophyll
b = Phycocyanin

Fig. 13

Plate 11 Effects of sodium chloride levels (a = control and b=20 mM) on the chlorophyll fluorescence of *Anabaena azollae* (900 x)

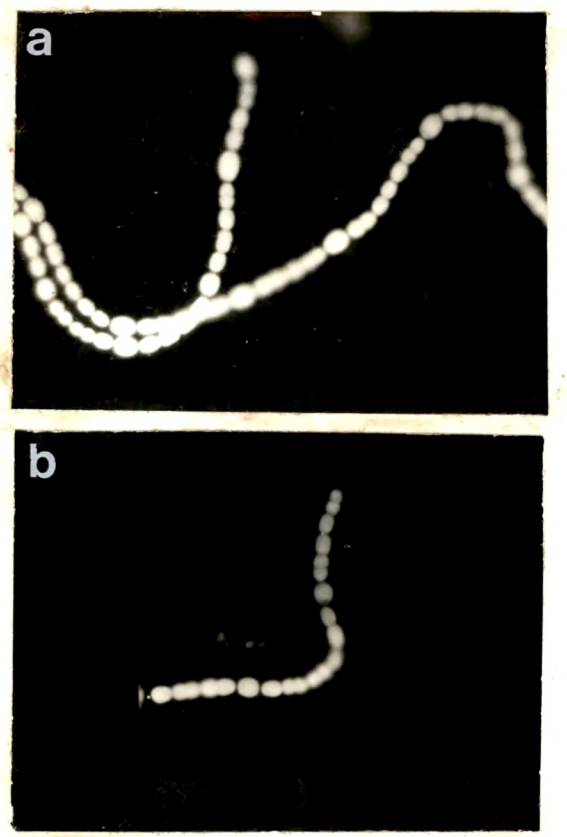

Plate 11

Plate 12 Effects of sodium chloride levels (a = control, b=10, c=20 and d=40 mM) on the phycocyanin fluorescence of *Anabaena azollae* (900 x)

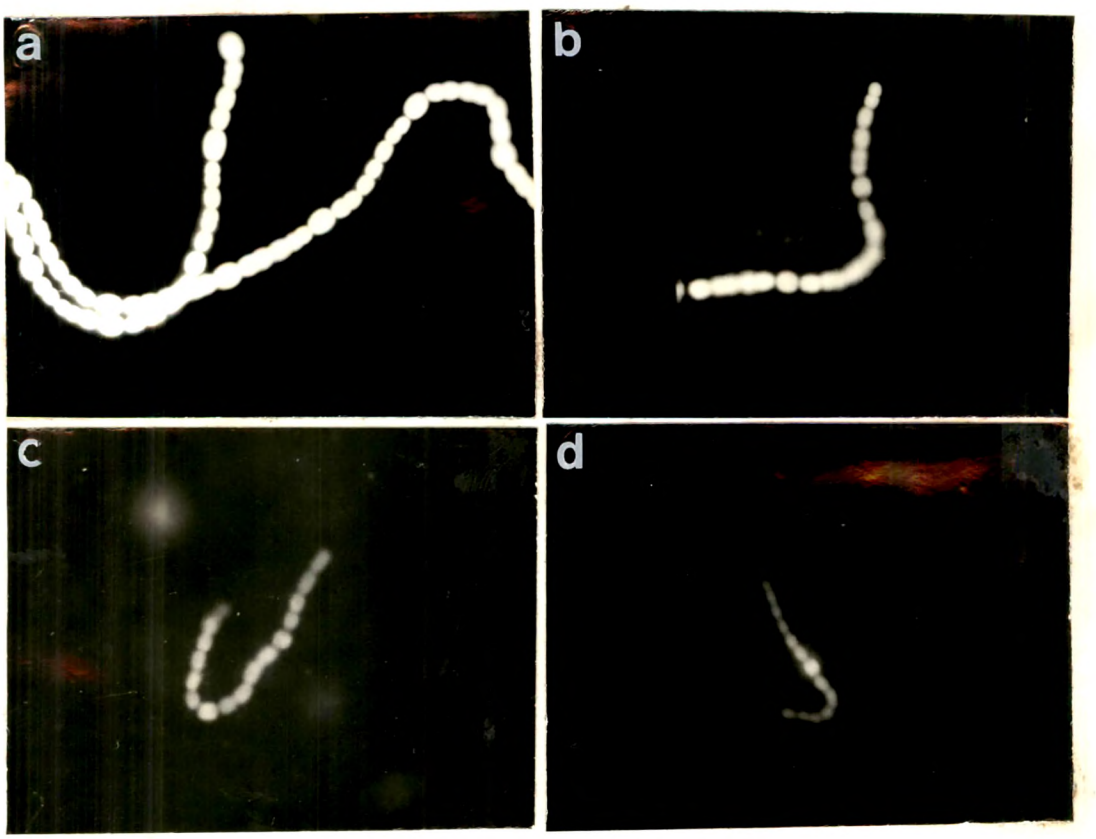

Plate 12