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THE GROWTH AND NITROGEN FIXATION OF AZOLLA

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Azolla is a genus of water fern that assimilates nitrogen in association with nitrogen fixing blue green alga, *Anabaena azollae* which lives in the cavities present in the upper leaf lobes of azolla as symbiont (Moore, 1969). *Azolla* has attracted the attention of rice scientists for its utilization in rice production. Biological nitrogen fixation through *Azolla—Anabaena* complex is considered to be a potential biological system for increasing rice yield at comparatively low cost (Kannaiyan, 1978). The biology (Moore, 1969), physiology (Peters *et al.*, 1980) and utilization of azolla for rice (Watanabe, 1978) have been studied in detail. The aquatic fern azolla has potential as green manure for low land rice because of itshigh relative growth rate (Moore, 1969). This work was initiated to compare the growth and nitrogen fixation abilities of six cultures of azolla and the results are presented.

Materials and Methods

Azolla pinnata (Coimbatore), *Azolla fillculoides* (Italy), *Azolla microphylla* (Galapagos Islands), *Azolla mexicana* (California), *Azolla* sp. (Paraguay) and *Azolla* sp. (Brazil) maintained in the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore were used in this study.

Growth studies

Soil extract solution was prepared and used as basal medium. One litre of soil extract solution was taken in plastic tubs and fresh azolla inoculum from the six cultures mentioned above were inoculated into the plastic tubs at 1 g quantity. The tubs were maintained under green house condition and the biomass was recorded on 10th day.

Nitrogenase activity

A constant weight of 2g fresh azolla was taken for all the cultures. Azolla fronds were blotted dry and placed in 50 ml flask and acetylene injected into the flask was equal to ten per cent of the total volume. After an incubation period of 3 h one per cent of the gas mixture was withdrawn and injected into a Perkin Elmer gas chromatograph for the estimation of ethylene. From this the nitrogenase activity was calculated and expressed as n Moles of ethylene produced per hour per gram dry weight of azolla based on the method developed by Hardy *et al.* (1968).

Chlorophyll content

Chlorophyll contents were estimated by following the method described by Mahadevan and Sridhar (1982).

^{14}C fixation

The amount of labelled ^{14}C liberated from labelled barium carbonate fixed by azolla was estimated based on the method developed by Andrews and Hornsey (1972).

NPK and micronutrient analysis

Azolla samples were dried and powdered. The total nitrogen was estimated by microkjeldahl method in a kjeltec autoanalyser as described by Humphries (1956). The total phosphorus content was estimated by triple acid digestion method as detailed by Jackson (1958). Potassium was estimated from the triple acid extract using a flame photometer (Jackson, 1958). Micronutrients were analysed by following the method of Lindsay and Norwell (1969).

Ammonia excretion

Ammonia content of azolla was estimated on 5th, 10th and 15th day in a Shimadzu double beam U. V. spectrophotometer at 410 nm following the method of Sahrawat and Prasad (1975).

Ammonia assimilating enzymes

The glutamine synthetase (GS) activity was estimated by following the method of Betteridge and Ayling (1976). The glutamate synthase activity (GOGAT) was measured as per the method developed by Vandescasteel *et al.* (1975) and glutamate dehydrogenase activity (GDH) was estimated as per the method of Doherty (1970).

Results and Discussion

Species delineation in *Azolla* is based primarily upon reproductive structures. The genus *Azolla* comprises four new world species in the subgenus *Euazolla* and two old world species in the subgenus *Rhizosperma* (Moore, 1969). The *Euazolla* species include *A. caroliniana* Willd., *A. filiculoides* Lamark, *A. mexicana* Presl. and *A. microphylla* Kaulfuss. *A, pinnata* R. Brown and *A. nilotica* De Caisne comprise the subgenus *Rhizosperma.* These species are widely distributed in tropical and temperate fresh water ecosystems throughout the world (Kannaiyan, 1978). Among the six different cultures tested, *A. microphylla* (Galapagos Islands), *Azolla* sp. (Brazil) and *A. pinnata* (Coimbatore) have recorded maximum biomass yield (Table 1). *A. filiculoides* (Italy) recorded poor growth. However, it is interesting to note that *A. feliculoides* (Italy) recorded highest nitrogenase activity followed by *Azolla* sp. (Brazil) while *A. mexicana* (California) and *Azolla* sp. (Paraguay) have recorded poor nitrogenase activity (Table 1). *Azolla pinnata* is Known to multiply well under tropical climatic conditions particularly at 30°C and *A. filiculoides* is a temperate culture which normally multiplies under low temperature of about 25°C (Peters *et al.* 1980). Kannaiyan (1984) studied five azolla cultures under 25/15°C day/night temperature with 250 $\mu E/m^2/S$ and found *A. filiculoides* (Hawaii) and *Azolla* sp. (Paraguay) have

recorded higher biomass. The poor growth of *A. filiculoides* in the present study might probably due to higher temperature prevailed during the experimental period i. e., 30 + 1°C. Talley and Rains(1980) showed that *A. filiculoides* recorded higher nitrogenase at high temperature of 35–40°C. It is difficult to explain the mechanism involved in *A. filiculoides* for the induction of nitrogenase at higher temperature.

The chlorophyll content was maximum in *Azolla* sp, (Brazil) and *Azolla* sp. (Paraguay) and low in *A. pinnata* (Coimbatore) and *A. filiculoides* (Italy) (Table 2). Chlorophyll 'a' was maximum in *Azolla* sp, (Brazil) and chlorophyll 'b' was maximum in *Azolla* sp. (Paraguay). *Azolla* chloroplasts contain chlorophyll 'a' and chlorophyll 'b', the *Anabaena* filaments contain chlorophyll 'a' and phycobiliproteins and both contain carotenoides (Tyagi *et al.*, 1980). They also found that *in vivo* phycocyanin accounts for about 70 per cent of the total phycobiliproteins while phycoerithrocyanin and allophycocyanin account for about 17 per cent and13 per cent respectively. As in free living cyanobacteria (Stewart, 1980) there is a close relationship between photosynthesis and nitrogen fixation in the *Azolla—Anabaena* association (Peters *et al.* 1976). An experiment with ^{14}C labelled CO_2 fixation in six cultures of Azolla revealed the higher ^{14}C labelled CO_2 fixation in *Azolla* sp. (Brazil) and *Azolla* sp. (Paraguay) and low in *Azolla pinnata* and *A. filiculoides* (Table 3). This result confirms the chlorophyll study where the total chlorophyll accumulation was significantly more in *Azolla* sp. (Brazil) and *Azolla* sp. (Paraguay). Appreciable information is available on nitrogen fixation and related processes as well as for photosynthetic carbondioxide fixation in the intact symbiotic association (Peters *et al.*, 1976). Action spectra of acetylene reduction in the association and isolated endophyte have further demonstrated the interaction of photosynthesis with nitrogen fixation (Tyagi *et al.*, 1980). The results of the present investigation have lucidly indicated the accumulation of chlorophyll in *Azolla* sp. (Brazil) and *Azolla* sp. (Paraguay) which has got practical significance under field conditions in increasing the nitrogen fixing potential.

Total N content was maximum in *A. filiculoides* (Italy) and *Azolla* sp. (Brazil) followed by *A. pinnata* (Coimbatore) and *A. microphylla* (Galapagos Islands). *A, mexicana* (California) and *Azolla* sp. (Paraguay) recorded low N content (Table 4). Phosphorus was significantly more in *A. pinnata* (Coimbatore) and in *Azolla* sp. (Brazil). *A. mexicana* (California) recorded low values for P. *A. filiculoides* (Italy) has recorded higher K content when compared to other cultures. However, there was not much variation in K content between the cultures tested.

Micronutrient studies revealed that the iron content was significantly more in all the cultures (Table 5). Iron accumulation was comparatively high in *A. pinnata* (Coimbatore) followed by *A. filiculoides* (Italy). The Cu and Zn content was maximum in *A. mexicana* (California). In general accumulation of Cu, Fe and Zn was relatively more in *A. filiculoides* (Italy) compared to other cultures studied.

Table 1

The growth and nitrogenase activity of six cultures of *Azolla*

Azolla cultures	Biomass (g)	Nitrogenase activity (n moles of C_2H_4 produced/h/g)
Azolla pinnata		
Coimbatore	1.92	160.00
Azolla filiculoides		
Italy	1.70	800.00
Azolla microphylla		
Galapagos Islands	2.21	186.67
Azolla mexicana		
California	1.87	80.00
Azolla sp.		
Paraguay	1.78	86.67
Azolla sp.		
Brazil	1.95	320.00
SE	0.055	857
SED	0.078	12.12
CD (0.05)	0.166	25.46

Table 2

The chlorphyll content of six cultures of *Azolla*, mg/g

Azolla cultures	Chlorophyll 'a'	Chlorophyll 'b'	Total chlorophyl
Azolla pinnata			
Coimbatore	0.165	0.061	0.226
Azolla filiculoides			
Italy	0.165	0.061	0.226
Azolla microphylla			
Galapagos Islands	0.223	0.016	0.239
Azolla mexicana			
California	0.267	0.021	0.288
Azolla sp.			
Paraguay	0.268	0.115	0.383
Azolla sp.			
Brazil	0.319	0.080	0.399
SE	0.005	0.001	0.004
SED	0.007	0.002	0.005
CD (0.05)	0.015	0.004	0.011

Table 3

The fixation of ^{14}C labelled CO_2 by six cultures of *Azolla*

Azolla cultures	^{14}c Cps/g
Azolla pinnata Coimbatore	432
Azolla filiculoides Italy	464
Azolla microphylla Galapagos Islands	480
Azolla mexicana California	494
Azolla sp. Paraguay	504
Azolla sp. Brazil	512

Table 4

The NPK contents of six cultures of *Azolla*, per cent

Azolla cultures	N	P	K
Azolla pinnata Coimbatore	3.17	1.11	1.23
Azolla filiculoides Italy	5.19	0.88	1.41
Azolla microphylla Galapagos Islands	3.03	0.83	1.31
Azollamexicana California	2.42	0.70	1.28
Azolla sp. Paraguay	2.20	0.75	1.23
Azolla sp. Brazil	4.29	1.06	1.03
SE	0.060	0.008	0.215
SED	0.085	0.011	0.304
CD (0.05)	0.178	0.024	0.637

Table 5

The micronutrient contents (ppm) of six cultures of *Azolla*

Azolla cultures	Mn	Cu	Fe	Zn
Azolla pinnata Coimbatore	2.22	0.21	53.79	1.54
Azolla filiculoides Italy	14.18	0.63	34.99	3.41
Azolla microphylla Galapagos Islands	16.12	0.42	26.66	2.94
Azolla mexicana California	18.07	0.42	25.82	2.93
Azolla sp. Paraguay	16.40	0.42	21.66	2.74
Azolla sp. Brazil	13.90	0.21	22.49	2.26

Table 6

The ammonia excretion by six cultures of *Azolla*, ppm

Azolla cultures	5th day	10th day	15th day
Azolla pinnata Coimbatore	1.8	1.7	1.5
Azolla filiculoides Italy	1.2	1.2	1.6
Azolla microphylla Galapagos Islands	0.8	2.2	2.4
Azolla mexicana California	0.6	1.0	2.6
Azolla sp. Paraguay	1.4	2.0	2.4
Azolla sp. Brazil	2.0	1.2	1.0
SE	0.144	0.150	0.139
SED	0.204	0.212	0.197
CD (0.05)	0.435	0.452	0.420

Table 7

Ammonia assimilating enzymes of six cultures of *Azolla* (enzyme units/mg of protein)

Azolla cultures	GS	GOGAT	GDH
Azolla pinnata			
Coimbatore	234.26	161 81	9.86
Azolla filiculoides			
Italy	229.66	109.84	20.45
Azolla microphylla			
Galapagos Islands	206.03	151.77	13.01
Azolla mexicana			
California	159.54	145.85	9 86
Azolla sp.			
Paraguay	220.66	195.06	13.93
Azolla sp.			
Brazil	276.86	201.32	17·04
SE	4.37	3.19	0.541
SED	6.18	4.51	0.765
CD (0.05)	13.17	9.61	1.630

The deficiency of Fe, Mn, Co, Zn, Cu, Mo and B had unfavourable effects on growth and nitrogen fixation in *A pinnata* (Yatazawa *et al.*, 1980). It has been found that Fe is the most limiting micronutrient to azolla growth often in natural rice field situations. The present study revaled the fair amount of micronutrient accumulation in the sporophytes of azolla which in turn might be made available to rice plant.

A. mexicana (California) recorded maximum ammonia excretion followed by *A. microphylla* (Galapagos Islands). Ammonia excretion was maximum on 15th day in all the cultures except in *A. pinnata* (Coimbatore) and *Azolla* sp. (Brazil). *A. pinnata* (Coimbatore) and *Azolla* sp. Brazil excreted maximum on 5th day (Table 6). The amount of ammonia in the medium could be attributed to the exogenous excretion of the sporophyte as well as the liberation of decaying roots shed off from the fronds. The excretion of ammonia by *A. mexicana* has been demonstrated by Peters (1976). This needs critical further study under flood water situation in rice field to pin point the amount released by azolla and from that the amount taken by rice plant.

Among the six azolla culture *Azolla* sp. (Brazil) recorded maximum GS and GOGAT activity while *A. filiculoides* recorded highest GDH activity and lowest GOGAT activity (Table 6). *A. mexicana* recorded lowest GS and GDH activity. Both partners exhibit GS, GOGAT and GDH activities. However, it has been

estimated that about 90 percent of the associations total GDH activity are attributable to the azolla (Peters *et al.*, 1979). In accord with the release of newly fixed N_2 as ammonia, GDH with its lower affinity for ammonia may regulate the utilization of ammonia by the symbiont. In the present study *Azolla* sp. (Brazil) and *Azolla* sp. (Paraguay) have registered higher GS and GOAT activities. This results suggest a nitrogen limiting condition or a lower concentration of ammonia has shifted the pathway of ammonia assimilation to the GS—GOGAT pathways which normally operate in lower concentration of ammonia (Yates and Eady, 1979). The present results clearly revealed the potentiality of the azolla cultures in possessing the ammonia assimilating enzymes for ammonia assimilation primarily in the cell components of azolla sporophytes.

Summary

Among the six azolla cultures studied *A. microphylla* (Galapagos Islands) and *Azolla* sp. (Brazil) have recorded maximum growth rate. The highest nitrogenase activity was recorded in *A. filiculoides* (Italy) followed by *Azolla* sp. (Brazil). Nitrogen content was also more in these two spacies of *Azolla.* The chlorophyll 'a' accumulation was significantly more in *Azolla* sp. (Brazil) whereas chlorophyll 'b' was more in *Azolla* sp. (Paraguay). *Azolla* sp. (Brazil) has also registered higher GS, GDH and GOGAT activities followed by *Azolla* sp. (Paraguay) Ammonia excretion was found to be maximum on 15th day after inoculation in *A. mexicana* (California) followed by *A. microphylla* (Galapagos islands). The micronutrients status of Mn, Cu, Fe and Zn was investigated and iron content was significantly more in all the azolla cultures. Iron content was maximum in *A. pinnata* (Coimbatore) while *A. mexicana* (California) has recorded higher Mn content. *A. filiculoides* (Italy) has recorded higher Cu and Zn contents. In general, it was noticed that the accumulation of Cu, Fe and Zn was relatively more in *A. filiculoides* (Italy) compared to other cultures.

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