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MEMORANDUM

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STUDIES ON THE EFFECT OF TEMPERATURE ON THE GROWTH AND NITROGEN FIXATION IN FOUR STRAINS OF *AZOLLA PINNATA* R. BROWN*

S KANNAIYAN AND C SOMPORN**

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY, TAMIL NADU AGRICULTURAL UNIVERSITY, COIMBATORE 641003

Azolla strain A. pin-GP-BT and A. pin-BR-M have recorded higher biomass in both the incubation periods while A. pin-SK-I and A. pin-SK-CI have recorded poor growth. The relative growth rate (RGR) was low on 27th day in all the four strains compared to 19th day. Nitrogenase activity was comparatively more in A. pin-SK-I and A. pin-BR-M on 19th day. On 27th day the nitrogenase activity was completely inhibited in A. pin-GP-BT and A. pin-SK-CI. Both A. pin-BR-M and A. pin-GP-BT have recorded more chlorophyll contents while A. pin-SK-I and A. pin-SK-CI have registered low chlorophyll accumulation. Nitrogen accumulation was more in A. pin-GP-BT and A. pin-BR-M.

Azolla is a water fern that assimilates atmospheric nitrogen in association with nitrogen fixing blue green alga-*Anabaena azollae* (Becking 1979). It is a potential biological system as biofertilizer for rice production (Lumpkin and Plucknett 1980). The growth of *Azolla* is largely influenced by various environmental factors (Becking 1979). Temperature is one of the most important environmental factors because the utilization of *Azolla* in the tropics is sometimes restricted by its low tolerance to high temperature (Watanabe and Berja 1983). The growth of *Azolla pinnata* was found to be maximum during winter months when the temperature was 25±1°C/17±1°C day/night cycle and these low temperature also known to induce the sporulation in *A. pinnata* (Kannaiyan 1982). The selection of high temperature tolerant strains of *A. pinnata* has therefore got potential for cultivation of *Azolla* as biofertilizer for rice in tropics. The effect of temperature (38±1°C) on growth, nitrogen fixation, chlorophyll accumulation and C, H N contents of four strains of *A. pinnata* was investigated and presented.

MATERIALS AND METHODS

An experiment was carried out to select high temperature tolerant strains of *Azolla pinnata*. *Azolla* strains *viz.*, A. pin-GP-BT, *A. pinnata*-Thailand-GP-BT, A. pin-BR-M, *A. pinnata*-Malaysia-BR-M, A. pin-SK-I *A. pinnata*-Sri Lanka SK-I and A. pin-SK-CI *A. pinnata*-India-SK-CI were used in this study.

* The study was carried out at C F Kettering Research Laboratory, Yellowsprings, Ohio 45387, USA.

**Present address : Division of Soil Science, Department of Agriculture, Banghen, Bangkok, Thailand.

Nitrogen free growth medium *i.e.* IRRI(-) NO_3 (Watanabe *et al.* 1977) was prepared and 150 ml was added in 500 ml wide mouth conical flasks and sterilized. Fresh *Azolla* fronds (0·2 g) were inoculated into the flasks and incubated in the glass house at 38±1°C/25±1°C day/night temperature cycle at 350 $\mu E\ m^{-2}\ S^{-1}$ light intensity for 27 days. The biomass, nitrogenase activity and chlorophyll content were determined on 19th and 27th day. After final determination of biomass on 27th day the fronds were dried in an oven at 60°C for 48 hr and the samples were powdered and used for CHN analysis.

The growth rate of the fern *Azolla* was measured on 19th and 27th day by recording the biomass yield. The doubling time was calculated by following the formula of Subudhi and Watanabe (1981). Doubling time=t/r where t=Experiment period, $r=\log \frac{(W1-W0)}{0{\cdot}301}$ Where W1=weight after 't' days, W0=weight of initial inoculum, 0·301=constant. The relative growth rate (RGR) is 0·693/doubling time, where 0·693 is a constant factor.

Acetylene reduction assay for estimating the nitrogenase activity was done following the methods of Peters and Mayne (1974). *Azolla* fronds (0·1 g f wt) were incubated for 30–60 min under an atmosphere of 10% C_2H_2 : 0·03% CO_2 in argon in sealed vials using the IRRI(-) NO_3 medium. Sealed vials were incubated under 26±1°C temperature and 100 $\mu E\ m^{2}\ S^{-1}$ light intensity employed for the growth. Ethylene was measured by using a Varian 3740 FID gas chromatograph equipped with a Porapak N (80–100 mesh) column, 6 ft × 1/8 in. Column temperature was maintained at 90°C, and nitrogen was used as the carrier gas. Peak areas were measured utilizing a Hewlett-Packard 3390 A recording integrator. The nitrogenase activity was expressed as n. moles C_2H_4/min, per gram fresh weight.

For chlorophyll determination, *Azolla* fronds (0·1 g f wt) were extracted in 10 ml of 95% ethyl alcohol for a minimum of 16 min in the dark and centrifuged at 2500 rpm for 10 min and measured in a Varian DMS-90 UV-Vis spectrophotometer at ABS 665 and ABS 649 for determining chlorophyll was also counter checked by measuring at ABS 654. The chlorophyll content was expressed as micrograms per gram fresh weight. The total chlorophyll, chlorophyll-a, chlorophyll-b and chlorophyll a, b ratio was calculated following the formula of Wintermans and Demots (1965).

For carbon, hydrogen and nitrogen analysis, *Azolla* fronds were dried in an oven at 60°C for 48 h. Dried plant material was pulverized and used for carbon, hydrogen and nitrogen analysis. These analysis were determined with a Perkin-Elmer Model 240 Elemental Analyser equipped with a control Equipment Corporation MC-341-HA micro injector using 2·5 mg samples of the dried material weighed on a Cahn Model G electrobalance. Dinitrodurene was used as a standard. The C, H, N content of *Azolla* fronds was expressed as percentage of dry weight.

RESULTS

The strain A. pin–GP–BT and A. pin–BR–M have recorded higher biomass in both the incubation periods and thereby indicating their tolerance to high temperature regime (Table 1). A. pin–SK–I and A. pin–SK–CI have recorded poor growth in both the incubation periods. The relative growth rate (RGR) was low on 27th day in all the four strains compared to 19th day. The doubling time was low and relative growth rate was more for A. pin–GP–BT when compared to other strains tested.

Nitrogenase activity was comparatively more in A. pin–SK–I and A. pin–BR–M on 19th day while the activity was drastically reduced on 27th day (Table 1). Nitrogenase activity was completely inhibited on 27th day in A. pin–GP–BT and A. pin–SK–CI. The results indicated that the increase in the incubation period have inhibited the nitrogenase activity in all the strains tested.

TABLE 1. Growth of four strains of *Azolla pinnata* using a 38±1°C day and 25±1°C night regime.

Azolla strains	19th Day				27th Day			
	Biomass (g f wt)	Doubling time (Days)	RGR (g/g/day)	Nitrogen activity (n. moles C_2H_4/g fw min)	Biomass (g f wt)	Doubling time (Days)	RGR (g/g/day)	Nitrogen activity (n. moles C_2H_4/g fw min)
A. pin–GP–BT	1·85	5·97±0·17	0·116±0·003	2·48	2·32	7·64±0·05	0·091±0·001	—
A. pin–BR–M	1·62	6·32±0·21	0·110±0·004	5·33	1·78	8·56±0·05	0·081±0·001	0·58
A. pin–SK–I	1·22	7·42±0·40	0·094±0·050	6·32	1·56	9·12±0·23	0·076±0·002	0·40
A. pin–SK–CI	1·32	7·08±0·08	0·098±0·001	2·57	1·47	9·44±0·74	0·074±0·006	—

The chlorophyll-a, -b and total are given in Table 2. A. pin – BR–M and A. pin – GP–BT have recorded maximum total chlorophyll in both the incubation periods. Both chlorophyll-a and -b contents were significantly higher in A. pin – GP–BT and A. pin – BR–M compared to other strains tested. On 27th day chlorophyll-a was affected considerably in A. pin – SK–I. In general, chlorophyll contents were low in A. pin – SK–I and A. pin – SK–CI during both the incubation periods.

Nitrogen content was more in A. pin – BR–M and A. pin – GP–BT (Table 3). There was not much difference in carbon and hydrogen contents between strains tested. It is evident from the results that the high temperature regime 38 ± 1°C/25 ± 1°C day/night did not affect the nitrogen content very much in A. pin – GP–BT and A. pin – BR–M.

TABLE 2. Chlorophyll content of four strains of *Azolla pinnata* grown using a 38±1°C day and 25±1°C night regime.

Azolla strains	Chlorophyll contents (μ g/g f wt)							
	19th Day				27th Day			
	Chl a	Chl b	Total Chl.	Chl a, b ratio	Chl a	Chl b	Total Chl.	Chl a, b ratio
A. pin-GP-BT	84·61	24·27	108·88	3·49	97·03	29·36	126·39	3·31
A. pin-BR-M	99·64	29·91	127·55	3·57	96·51	30·10	126·61	3·21
A. pin-SK-I	68·75	19·51	88·25	3·52	33·10	34·53	67·63	0·96
A. pin-SK-CI	67·66	18·98	86·64	3·57	86·52	25·92	112·44	3·34

TABLE 3. CHN analysis of four strains of *A. pinnata* grown using 38±1°C day and 25±1°C night cycle.

Azolla strains	Temperature (38±1°C/25±1°C Day/Night)			
	C (%)	H (%)	N (%)	C : N ratio
A. pin-GP-BT	42·77	6·38	4·67	9·16
A. pin-BR-M	44·14	6·57	5·82	7·58
A. pin-SK-I	41·65	6·01	3·52	11·83
A. pin-SK-CI	42·26	6·02	3·77	11·21

DISCUSSION

In the present study the two strains of *A. pinnata viz.*, A. pin – GP-BT and A. pin – BR-M have shown tolerance to high temperature at 38±1°C/25±1°C day/night regime. Interestingly these two *Azolla* cultures have recorded more biomass during the growth period due to their tolerant nature to high temperature condition. The most favourable temperature for the growth and nitrogen fixation by *A. pinnata* is between 20 and 30°C (Lumpkin and Plucknett 1980). The optimum temperature for *A. pinnata*, *A. mexicana* and *A. caroliniana* when grown under 15 Klux artificial light was about 30°C while *A. filiculoides* required 25°C (Peters *et al* 1980). The present results suggest that the *Azolla* strain A. pin – GP-BT could tolerate high temperature. Kannaiyan (1982) found that the growth of *A. pinnata* was good at September to February under field conditions when the temperature was 24±1 to 28±1°C while the growth was drastically affected in summer months of March to June when the temperature prevailed from 35±1 to 39±1°C.

Nitrogenase activity was relatively higher in A. pin–BR–M and A. pin–SK–I on 19th day. These cultures registered very low activity on 27th day. Tung and Watanabe (1983) found that heterocyst frequency of the endophyte *Anabaena azollae* was low at 37/29°C day/night temperature. They have speculated that the diminished rates of nitrogenase in high temperature may have been effected by the differentiation of heterocysts. Becking (1979) investigated the nitrogenase activity of *A. pinnata* and *A. filiculoides* at 29°C and 33°C and found that there was difference in the nitrogenase activity between the temperature.

It is seen from the data that the chlorophyll a, b and total were higher in A. pin – BRM and A. pin - GP–BT. The results suggest that high temperature has got definite influence on the chlorophyll reduction in A. pin – SK–I and A. pin – SK–CI. *Azolla* chloroplasts contain chlorophyll-a and chlorophyll-b, the *Anabaena* filaments contain chlorophyll-a and phycobiliproteins and both contain carotenoids (Tyagi *et al.* 1980). As in free living cyanobacteria (Stewart 1980), there is a close relationship between photosynthesis and nitrogen fixation in the *Azolla – Anabaena* association (Peters and Mayne 1974). In the present study it is interesting to note that even under the high temperature at 38±1°C stress condition the strains A. pin – BR–M and A. pin – GP–BT have the ability to synthesize chlorophyll. Photosynthesis and nitrogen fixation are inter-related process in the *Azolla-Anabaena* symbiosis. Photosynthesis in the endo-symbiont alga in *Azolla* produces the reductant utilized for nitrogen fixation and this was established by the findings that phycocyanin is concentrated in algal vegetative cells adjacent to the heterocysts (Becking 1979).

Nitrogen content was more in A. pin – GP–BT and A. pin – BR–M. Watanabe and Berja (1983) found that the nitrogen content was 15 g N m^{-2} by *A. caroliniana* at 22°C for 41 days while the same culture produced 6·3 g N m^{-2} at 29°C for 28 days. The *Azolla* strains A. pin – GP–BT and A. pin – BR–M have potential under high temperature rice growing areas in tropics.

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