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Growth Characteristics, Heterocyst and Spore Differentiation in the Endosymbiotic Cyanobacterium *Anabaena cyeadeae*

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***Anabaena cycadeae*, isolated locally from *Cycas coralloid* roots in clonal and axenic cultures, is capable of photoautotrophic, photoheterotrophic, photomixotrophic and heterotrophic diazotrophic growth. Cultures grown in DCMU with or without Na_2S showed no photosynthetic growth but when grown in presence of sulphide (reducing condition) showed better growth than culture grown aerobically. The enhanced growth in Na_2S may be because of sulphide, acting simultaneously with water as source of electron. It is interesting to mention that DCMU or DCMU with Na_2S treated N_2-culture showed dominance of water soluble pigment (phycocyanin). This indicates that cultures grown under this condition do not starve for endogeneous nitrogen reserves. A slight increase in heterocyst differentiation was observed under reducing conditions. In all cases spore initiation always occured from mid cells between two heterocyst and subsequently extended towards either of heterocyst resulting into a chain of spore and took 22-26 days to develop spore. Heterocyst frequency increased from 8-14% in reducing condition and reduced drastically in DCMU treated culture. Addition of glucose to DCMU cultures, protected heterocyst inhibition while no heterocysts were observed in DCMU and Na_2S supplemented medium. The level of total nitrogen increased in cultures grown in glucose supplemented DCMU medium with increase in heterocyst frequency under similar growth condition.**

Key words: DCMU, Na_2S, Growth, Heterocyst, Spore, *Anabaena cyeadeae*.

Cyanobacteria occur in the symbiotic association more frequently than any other groups with possible exception of chlorophyceae. The association between waterfern *Azolla* and cyanobacterium *Anabaena* is found in temperate and tropical ecosystems throughout the world. The potential of *Azolla-Anabaena* association as a nitrogen source in agriculture especially in rice cultivation was indicated by Moore (1969) and has been substantiated by others (Whitton, 2000; Nayak *et al*. 2004; Pabby *et al*. 2003). The endosymbiotic cyanobacterium *Anabaena cycadeae* are localized in the distinct area of Cortex in cycades.

Cyanobacteria beeing aerobic, are also grouped as microaerophilic organism showing optimal growth in a low oxygen tenson. The microaerophilic nature of Cyanophytes was emphasised much earlier (Singh, 1950; Singh, 1978; Stewart and Pearson, 1970; Whitton, 2000). Reducing conditions when stimulated under laboratory, has favourable effect on several nitrogen-fixing cyanobacteria (Singh *et al*. 1972).

Cyanobacteria combine a prokaryotic cellular organisation with eukaryotic type of oxygenic photosynthetic system (Krogmann, 1973). The PS II can be inhibited by DCMU and under these conditions cyclic electron flow is

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maintained around PS I in a manner analogous to those of photosynthetic bacteria. The reduction is growth is always accompanied by decreased rate of photosynthesis and nitrogen assimilation (Niven, *et al*. 1987). It is now established that both unicellular and filamentous cyanobacteria can perform sulphide dependent anoxygenic photosynthesis (Oren *et al*., 1977; Padan, 1979; Kashyap, *et al*., 1983). In view of crucial role of sulphide on photosynthesis, the present investigation deals with the effect of sulphide on microaerobic growth, heterocyst and spore differentiation in endosymbiotic cyanobacterium *Anabaena cycadeae*.

MATERIAL AND METHODS

Maintainance of culture

The coralloid root of *Cycas revoluta* growing about 6-8 inches deep in the soil were removed, thoroughly washed in tap water to remove the adhering soil particles and finally sterilized by double distilled water. The roots were surface sterilized with 1% $HgCl_2$ solution (w/v) for 2 to 5 minutes followed by sterilization with double distilled water to remove trace of mercury from coralloid root. The outer epidermal layer was stripped off with the help of sterilized scalpel under aseptic condition and the blue-green zone was disseeted out and transferred on agar nutrient containing Allen and Arnon nutrient medium with A6 as microelement (Allen & Arnon, 1955). Plates were kept in the culture room under photoautotrophic conditions (temperature 25±2ºC, pH 8.5 and flurescent light intensity 22 lux and 14:10 light:dark rythum) for 2-3 weeks. Colonies formed on these plates, away from bacterial contamination, were picked up under binocular microscope and transferred into tubes containing liquid basal medium.

EXPERIMENTAL

Exponantially grown homogeneous suspenson, equivalent to 24 mg protein. 0.1 ml^{-1}, of *Anabaena cycadeae* was used as a source of the inoculum and inoculated into 100 ml flask containing 50ml freshly prepared N_2–medium supplemented with different concentrations of sodium sulphide (5-100 μgml^{-1}) for reducing growth conditions. pH of the medium was adjusted to 8.5 as usual. For creating an aerobic growth conditions a freshly prepared aquous solution of DCMU was added into the N_2–medium at desired concentrations (0.1 to 10 μgml^{-1}) with or without combination of glucose (40 µM) or Na_2S (50μgml^{-1}). Cultures grown under photoautotrophic, photoheterotrophic and heterotrophic conditions were assayed for growth.

Extraction and estimation of pigments

A known volume of homogeneous suspension of cyanobacterial culture (5 ml) was centrifuged, washed twice and suspended in 5 ml acetone (80 v/v) and incubated at 4ºC overnight for complete extraction of total solvent soluble pigment. This was followed by centrifugation and the resulting supernatant was used for chlorophyll *a* and carotenoides estimation. The remaining residue was subjected to extraction of phycobilin pigments by chilled water. After freezing and thrawing the suspension was recentrifuged to obtain water soluble pigment phycocyamin. Pigments were estimated and quantitified by using methods described by Allen (1968), Brody & Brody (1961), and Myers & Kratze (1955).

Estimation of total protein

Cyanobacteria sample was mixed with 1N NaOH and kept on boiling water bath for 5 minutes, then cooled in running water. A known amount of the above extract (0.5 ml) was used for protein estimation as described by Lowry *et al*. (1951) with bovine serum albumun as standard.

Estimation of total nitrogen

Total nitrogen was estimated by taking 10ml distilate and titrated against 0.005N H_2SO_4 as described for micro Kjeldahl method. Total nitrogen was quantitified by using 1ml H_2SO_4 equivalent to 0.07mg of nitrogen.

Heterocyst and spore frequency

Heterocyst frequency, expressed as percentage (%) was calculated by counting the total number of heterocyst divided by number of vegetative cell present in cyanobacterial filament. The data are based on the average of 25 independent samples. Similaraly, sporulation frequency was also determined.

Statistical analysis

Values presented are mean of six experiments with their respective ± S.E. The significance of the differences for various

experiments were analysed using the students t-test at the level of significance of P<0.01.

RESULTS

Aerobic growth of *Anabaena cycadeae* in graded concentrations of sulphide has been summarized in Fig. 1. Since pH of the medium (8.2) altered with the increasing concentrations of sulphide. The initial pH of sulphide supplemented medium was adjusted to 8.2 by using phosphate buffer. The cyanobacterial growth was much better than control upto 100 µgml^{-1} with optimal growth at 50 µgml^{-1} (Fig. 1). This indicates that the cyanobacterium prefers reducing conditions for luxurient diazotrophic growth. Addition of DCMU to N_2–cultures caused decrease in growth about 55% at 0.1 µgml^{-1} that of control while higher concentrations caused progressive decrease in growth with complete inhibition and lysis of the cells at 5 and 10 µgml^{-1} (Fig. 2A). Cultures grown in lower concentrations of DCMU (0.1-0.5 µgml^{-1}) showed dominance of blue pigment with 55% to 65% growth inhibition and appeared more bluish than control. Cyanobacterium grown in sulphide (50 µgml^{-1}) supplemented with different concentrations of DCMU also showed similar kind of growth inhibition as observed with DCMU alone. This demonstrates that anaerobic conditions does not favour photosynthetic growth of *A.cycadeae* (Fig. 2B). It was observed that the growth inhibition at lower concentration of DCMU (0.1 to 1 µgml^{-1}) was partially abolished by glucose but not recovered at higher concentrations (Fig. 3).

The pigment composition of the cyanobacterium under reducing and anaerobic conditions is given in Table 1. There was a drastic reduction in Chl. *a* and DCMU treated cultures against control. However, the amount of pigments including phycocyamin in sulphide grown culture was 187.51±1.85 µgml^{-1} than control 123.07±1.41 µgml^{-1}. Cultures grown in 0.1 µgml^{-1} of DCMU, no doubt, showed reduction in phycocyanin content from 123.07±1.41 µgml^{-1} to 24-61±0.95 µgml^{-1} but phycocyamin/Chl. *a* ratio was found several fold

Table 1. Pigment composition of *A. cycadeae* grown under different growth conditions

Medium	Chl. a µg.ml^{-1}	Carot µg.ml^{-1}	Phyco. µg.ml	Phyco/Chl. a
Control (N_2-medium)	5.430±0.51	3.84±0.62	123.07±1.41	22.66±0.33
N_2+Na_2S	6.23±0.01	4.37±0.75	187.51±1.85	30.90±0.50
N_2+DCMU	0.09±0.01	0.15±0.20	24.61±0.95	273.44±2.35
N_2+DCMU+Na_2S	0.09±0.05	0.20±0.05	24.25±1.12	269.44±0.4

Note: The values are the means of six experiments with their respective± S.E.
(P < 0.01 when compared with control)
The concentration of Na_2S was 50 µg ml^{-1} and DCMU was 0.1 µg ml^{-1}

Table 2. Heterocyst frequency and total nitrogen fixed by the cyanobacterium *A. cycadeae* grown under different growth conditions

Growth Medium	Heterocyst %	Total N_2-fixed mg N_2 100 ml^{-1} culutre
Control (N_2-medium)	8.0±1.50	3.102±0.850
N_2+Na_2S[a]	14.2±1.20	4.510±0.350
N_2+DCMU[b]	2.0±0.75	1.105±0.25
N_2+DCMU+Na_2S	0.0±0.00	0.256±0.060
N_2+DCMUb[c]+Glucose[d]	6.8±0.25	2.101±0.520

The values are the means of six experiments with their respective ± S.E. and significant at P<0.01 when compared with control.
[a] = 50 µgml^{-1}, [b] = 0.1 µgml^{-1}, [c] = 1 µgml^{-1}, [d] = 40 MM.

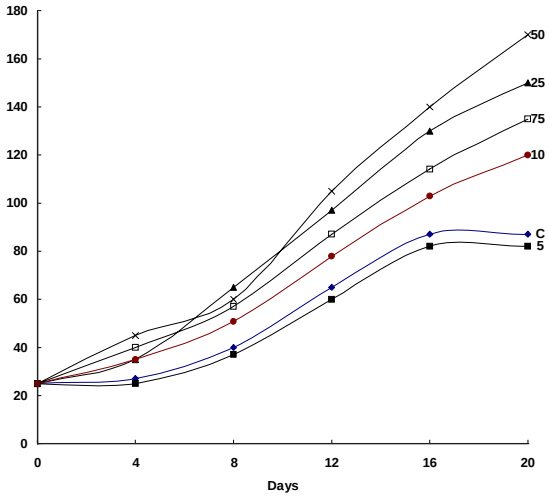

Fig. 1. Effect of different concentrations (mg/ml) of Na_2S on growth behaviour of *A. cycadeae*

Fig. 2. The effect of DCMU (A), and Na_2S (50 mg/ml) on nitrc

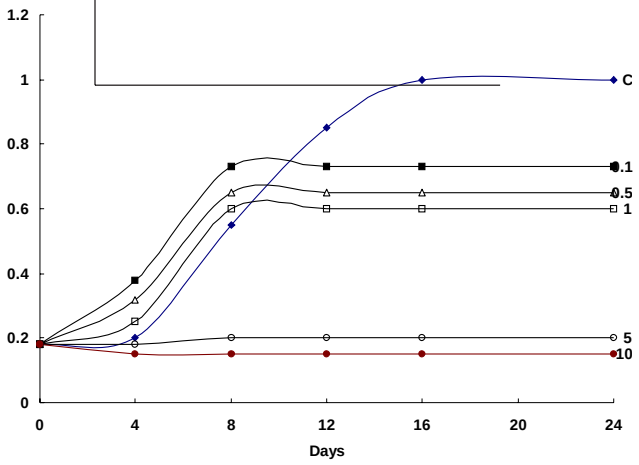

Fig. 3. Photohetrotrophic assimilation of glucose (40 mM) by *A. cycadeae* in the presence of different concentration (mg/ml) of DCMU

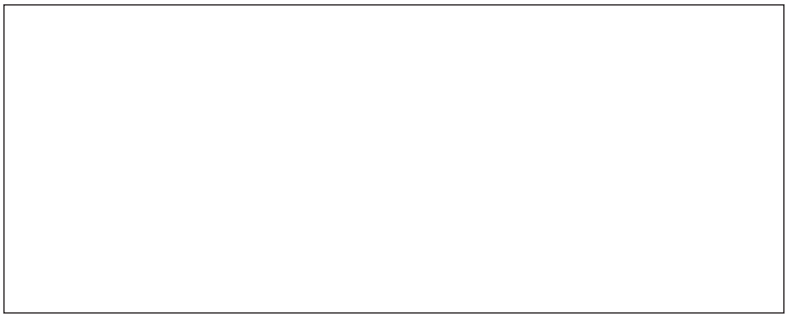

Fig. 4. Effect of Na_2S, DCMU and DCMU + Na_2S on heterocyst and spore differentiation in *A. cycadeae*

more than control (i.e. 273.44±2.35 against 22.66±0.33). Similar trend of pigment composition was observed with DCMU supplemented Na2S grown culture (Table 1). The results demonstrated that cultures do not starned for endogeneous stored nitrogen.

During cyanobacterial growth about 8.0±1.50% of vegetative cells were differentiated into heterocysts at a regular intervals. Heterocyst frequency increased from 8±1.50 to 14±1.20% in reducing conditions and decreased drastically in DCMU treated culture. Addition of glucose to DCMU cultures, protected heterocyst inhibition while no heterocysts were observed in DCMU and Na_2S supplemented medium (Table 2). Total nitrogen fixed by the cyanobacterium in 50 μgml^{-1} of Na_2S was 4.51±0.35 mg N. 100 ml^{-1} culture against control (3.102±0.850 mg N. 100 ml^{-1} culture). The level of total nitrogen increased in culture grown in glucose supplemented DCMU medium with increase in heterocyst frequency under similar growth condition (Table 2).

There is production of spore in stationary phase of the cyanobacterium in N_2–medium and spore production frequency was decreased with increasing concentration of Na_2S while heterocysts were increased (Fig. 4A). However, a totally reverse trend was observed in their differentiation on increasing concentrations of DCMU with and without 50 μgml^{-1} of Na_2S (Fig. 4B C). These observations suggest that heterocyst play no role in spore development. In all cases spore initiation always occurred from mid cells between two heterocyst and subsequently extended towards either side of heterocyst resulting into a chain of spores and took 22-26 days to develop formation from spore in N_2–medium.

DISCUSSION

The cyanobacterium was found to be facultative photoautotrophs since it also showed heterotrophic and photoheterotrophic growth in the presence of glucose with respect to dark and light conditions (Ojha, 1986). Several cyanobacteria have been reported to possess the duel role of nutrition (Fogg *et al*., 1973). The heterotrophic mode of nutrition at the cost of glucose suggests that the sugar must be acting as both carbon and energy sources for N_2-fixing growth (Rippka, 1972). The ability of the cyanobacterium to grow heterotrophically in absence of PS II, blocked by DCMU, suggests for the assimilation of glucose at the cost of solar energy.

Anoxygenic photosynthesis where sulphide replaces water at the source of ultimate electron donor, has been reported in the range of cyanobacteria (Cohen *et al*., 1975). The present observations are considered with the report of Stewart and Pearson (1970) that sulphide dependent N_2–growth of *N. cycadeae* indicates that photolysis of water by PS II system in essential for growth. Detailed investigation have shown that sulphide is oxidised to sulphur in a reaction solely drived by PS I (Garlick, 1977). However, the enhanced N_2–growth obtained in pH dependent sulphide medium does indicate that when photolysis of water is reduced but not completely,

sulphide may some simultaneously with H_2O as a source of electron or directly for N_2–fixing system. The electron derived by PS I from sulphide can be channalled either to CO_2 fixation, hydrogen evolution or N_2–fixation (Padan and Cohen 1982). This explains in the light, cyanobacteria in general grow more rapidly under microaerobic conditions than under fully aerobic conditions. The higher level of phycocyanin which principally joins PS II, in organism grown with sulphide perhaps indicates that sulphide may also be linked to PS II or an analogous system and meerely substitutes for water as electron donor (Cohen *et al*., 1975 a,b; Tiwari *et al*., 1982).

The enhanced frequency of heterocysts observed in field materials is due to the short filamentous morphology as has been reported by blending the N_2–cultures under laboratory conditions (Singh *et al*., 1972; Srivastava, 1985; Wolk, 1967). The reduced frequency of heterocyst in DCMU may be because of the limited carbon supply as exogeneous supply of glucose in DCMU medium abolished this indirect inhibition of heterocyst by DCMU. The enhanced level of total nitrogen fixed under reducing conditions may be due to the production of more heterocysts while decrease in total nitrogen in DCMU may be because of decreased heterocyst frequency. The level of total nitrogen increased in cultures grown in glucose supplemented DCMU medium with increase in heterocyst under similar conditions. Delay in sporulation period in Na_2S medium may be due to extenson of log phase growth while the reverse is true with DCMU. Spore production in the present system did not undergo massive dormancy phase but underwent germination in situ after 4 to 8 days in culture could be prevented from germination by simply incubating such sporulating cultures in dark.

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