Regulation of nitrogen fixation in *Cycas revoluta* and *Azolla pinnata* Association

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Abstract: Freshly collected water fern *Azolla* and coralloid roots of *Cycas* exhibited very high nitrogenase activity under aerobic conditions, yet no nitrate reductase activity. The nitrogenase activity of the Cycas coralloid roots and *Azolla* fronds was linear for 2 to 3h in both light and dark. However the activity was lost when the coralloid roots were suspended in water or a nitrogen free Allen & Arnnon. In contrast, the *Azolla* fronds exhibited a stimulated nitrogenase activity when kept in water or in nitrogen free Allen & Arnnon. NO_3 (5mM) or $NH4^+$ (5mM) had a negligible effect on the nitrogenase activity in the Azolla plants for up to 18h of incubation. It was interesting to note that the isolated phycobiont of *Azolla* showed a significant level of nitrate reductase under free-living conditions. Comparative studies of nitrogenase activity in free living isolate(s) of Azolla and coralloid roots of *Cycas* showed that laboratory grown *Anabaena azollae* was 100 times more efficient in terms of nitrogen fixation than *Anabaena cycadeae*. From the current results it was evident that nitrate reductase activity was absent in natural nitrogen fixing plants, whereas isolated symbiotic nitrogen fixing algae exhibited a differential efficiency in nitrogen fixation capacity.

1. Introduction

In symbiotic associations, other than those of legumes, an extensive range exists, including both higher and lower plants, although in every case a eucaryotic macro-symbiont (host) houses an N_2 fixing prokaryotic microsymbiont (endophyte). N_2 fixing cyanobacteria are important as they exhibit a symbiotic association ranging from gymnospermic plants to non-photosynthetic fungi. This type of association, which involves heterocyst-forming cyanobacteria, has received much attention because dinitrogen reduced by cyanobacteria serves as nitrogen source for the growth of a eucaryotic host (Stewart et. al., 1980; Stewart et. al., 1983; Haselkron and Buikema, 1992). Although detailed studies have been carried out on growth, N_2 fixation, and other physiological processes in various free-living cyanobacterial species (Stewart, 1980), little work has been carried out on symbiotic nitrogen fixation and its regulation (Stewart et. al. 1983; Stewart 1980; Meeks et. al., 1985; Bohme, 1998; Rai et. al., 2000). It is also pertinent to mention that recently the water fern *Azolla* has attracted much interest due to its potentials as a biofertilizer (Stewart et. al., 1983; Zimmerman, 1987). The aquatic fern *Azolla* is widely distributed in temperate and tropical freshwater and all species normally contain *Anabaena azollae* in the dorsal pores of the leaf loab. The endophyte remains associated with the Azolla through all stages of differentiation and development (Hill 1975; Hill 1977 & Peters and Mayne 1974). In contrast the gymnospermic plant *Cycas* widely distributed throughout the tropics and subtropics possesses coralloid roots, which contain cyanobacteria. The coralloid roots are infected by N_2 fixing *Nostoc* or *Anabaena* in the later stages of its development and the endosymbiont is able to fix dinitrogen even under free-living conditions (Caiola, 1974; Rai et. al., 2000).

Since both *Azolla* and *Cycas* grow in distinct ecological conditions, it is possible that the rate of N_2 fixation may also be significantly affected. Furthermore, little is known about the other factors, which may regulate N_2 fixation in these plants in nature. Accordingly, this study investigated the nitrogenase and nitrate reductase activity in *Azolla* plants and *Cycas* coralloid roots under standard conditions as well as the effects of certain factors in regulating nitrogenase activity were also studied.

2. Materials & Methods

The water fern *Azolla* was collected from a local pond on the Banaras Hindu University Campus. Healthy coralloid roots were collected from the *Cycas* plant growing in the Botanical garden at B. H. U. A single plant was chosen for the source of the coralloid roots used in all the experiments. Both the *Azolla* leaf

and the coralloid roots of *Cycas* were thoroughly rinsed with running water to remove all soil debris and then surface sterilized by immersion in 0.5%$HgCl_2$ for five minutes followed by repeated washing with sterilized distilled water. The endophyte (in root a green zone in the cortex) was teased out using sterilized needle and scalpel) and surface sterilized *Azolla* leafs were crushed in sterile normal saline and centrifuged at 5000 rpm for 10 minutes. The supernatant was streaked onto the nitrogen free Allen and Arnon (1955) medium: $\{(gl^{-1})\}$ $MgSO_4.7H_2O$ – 0.025; $CaCl_2$-0.05; NaCl – 0.20; K_2HPO_4 – 0.35; *A5 trace element stock solution – 1.0 ml; pH – 8.5-9.1; The *A5 trace element stock solution has following constituents (gl^{-1}): Boric acid -2.86: $MnCl_2$- 1.81; $ZnCl_2$- 0.222; Molybdenum trioxide (85%) – 0.018; Cupric sulphate – 0.079} and incubated in a culture room at 27±2ºC illuminated with day light fluorescent tubes (14.4 Wm^{-2} = 2400 lux) for 14h d^{-1}. The isolated endophyte was purified by streaking on nitrogen free Allen and Arnnon (1955) and maintained in axenic form in a culture room at 27±2ºC (Srivastava et. al., 1988). Cultures of *A. azollae* were grown routinely in a 0.1% agar supplemented nitrogen free Allen & Arnnon medium while *Anabaena cycadeae* isolated from coralloid roots of *Cycas revoluta* were grown routinely in a liquid nitrogen free Allen and Arnnon medium in a culture room at 27±1ºC illuminated with day light fluorescent tubes (14.4 Wm^{-2} = 2400 lux) for 14h d^{-1}. The chlorophyll a concentration was determined according to the method of Mackiney (1941).

The nitrogenase activity was measured using acetylene reduction technique as per the standard method of Stewart et. al., (1967). Two ml of a laboratory grown culture of algae or the desired amount of the *Azolla* plants or *Cycas* coralloid roots were placed in a 7ml vacutainer tube (Becton Dickinson, Rutherford, N. J., U. S. A.). All the assays were performed at 27ºC and 14.4 Wm^{-2} light intensity. The dark treatments were performed by wrapping the vials with aluminum foil.

The estimation of in vivo nitrate reductase was conducted following the method of Camm and Stein (1974). The activity was based on the amount of total nitrite formed, which was measured using the diazocoupling method developed by Lowe and Ewans (1964).

3. Results & Discussion

It is evident from Table-1 that the freshly collected Azolla plants and detached *Cycas* coralloid roots showed very high nitrogenase activity in both light and dark. The equal activity in light and dark was presumably due to the continuous supply of reductant by the host to the endophyte. It would appear that the host had a large pool of reductant that became available to the endophyte in the dark, as there was no alternative source of reductant since the endophyte lacked the capacity to fix CO_2 even though it included normal photosynthetic pigments (Ray et. al., 1979 & Tyagi et. al., 1981). Furthermore, the endophytes of the *Azolla* and *Cycas* roots had a very high heterocyst frequency i.e., 25 – 30%, which can also lead to high nitrogenase activity if the reductant and ATP are not limited. Table 1 shows that the nitrogenase activity of the *Azolla* plant was higher in double distilled water or the AA^- medium. An increase in nitrogenase activity following the incubation of *Azolla* plants with water or the nitrogen free Allen and Arnnon was expected because *Azolla* is adapted to an aquatic habitat. When the *Azolla* plants became devoid of water the general metabolic processes were affected due to water stress.

Table 1 Nitrogenase activity of intact *Azolla* plants and *Cycas* coralloid roots with and without liquid medium.

Conditions	Nitrogenase Activity[*]	
	Light	**Dark**
Only *Azolla* plants	0.73	0.56
Azolla plants + DDW[**]	0.84	0.76
Azolla plants + AA⁻ medium	0.93	0.85
Only Coralloid roots	0.95	0.87
Coralloid roots + DDW	0.08	0.72
Coralloid roots + AA⁻ medium	0.89	0.74

* Nitrogenase activity expressed in μmol $C_2H_4g^{-1}$f. wt. h^{-1}
** DDW - Double distilled water;
AA^- medium - Nitrogen free Allen & Arnon medium

Furthermore, the enhances nitrogenase activity, as observed in the nitrogen free Allen & Arnnon, was certainly due to the uptake of essential nutrients by the *Azolla* plants. These nutrients became limiting when the *Azolla* plants were incubated with the nitrogen free Allen & Arnnon, thereby affecting nitrogenase activity. In contrast the *Cycas* coralloid roots showed an abrupt loss in nitrogenase activity when suspended in double distilled water or the AA^- medium. However, this inhibition seemed to be temporary as the roots regained the initial level of nitrogenase as soon as they were taken out of the water or medium. This was due to the xerophytic characteristic of *Cycas*. Accordingly, water would appear to be inhibitory for the vital activities of coralloid roots. Figure-1 showed the nitrogenase activity of the isolated phycobiont of the *Azolla* and *Cycas* coralloid roots. *Anabaena azollae* showed a very high nitrogen fixing capacity, which was 100 times higher than the nitrogenase activity of *Anabaena cycadeae* under aerobic conditions. A rate

of 80 to 120 nmole C_2H_2 µg chl a^{-1} h^{-1} was routinely obtained in *A. azollae*. The observed rate of nitrogenase activity matches well with the growth rate of this cyanobacterium, which has a generation time of 8-12 hours. Furthermore, the higher nitrogenase activity also seemed to be correlated with a 15 – 20% heterocyst frequency. The nitrogenase activity and high heterocyst frequency separate this cyanobacterium from all other free-living cyanobacteria. The highest nitrogenase activity (108 nmole C_2H_2 µg chl a^{-1} h^{-1}) reported so far is from the marine cyanobacterium *Anabaena* CA (Gotto et. al., 1979; Herrer et. al., 2001) under optimum growth conditions with a generation time of only 4-5 hours. It is evident from Table-2 that there was no immediate and complete inhibition of nitrogenase activity in the intact Azolla plants by the addition of combined nitrogen sources like (NO_3^- or NH_4^+).

Table 2 Effect of combined nitrogen sources on nitrogenase activity of *Azolla*.

Conditions	Nitrogenase Activity*		
	18 h	24 h	48 h
Azolla plants + AA$^-$ medium	0.60	0.58	0.45
Azolla plants + AA$^-$ medium (NO_3^- 10mM)	0.63	0.33	0.14
Azolla plants + AA$^-$ medium (NH_4^+Cl 5mM)	0.61	0.33	0.17

*Nitrogenase activity expressed in µmol C_2H_4g^{-1}f. wt. h^{-1}; AA$^-$ medium - Nitrogen free Allen & Arnon medium

It would seem that the combined nitrogen sources were metabolized by the host and did not reach the endophyte. The current results are consistent with an earlier report (Zimmerman, 1987). The effect of combined nitrogen sources on the nitrogenase activity of the *Cycas* coralloid roots could not be studied because the liquid medium itself inhibited the nitrogenase activity. The failure of the combined nitrogen sources to completely inhibit the nitrogenase activity of the Azolla plants was interesting because under natural conditions there is also a presence of NO_3^- or NH_4^+. It should also be remembered that the concentration in the present investigation was much higher that the natural concentration of these ions.

It was interesting to find that the freshly collected *Azolla* and *Cycas* coralloid roots did not exhibit any nitrate reductase activity (Figure 2). However, when they were incubated with a higher concentration of KNO$_3$ the induction of nitrate reductase activity took place after 12 and 18 h in the *Cycas* root and *Azolla* plants respectively. The activity was significant and a gradual increase took place relative to the incubation time. However, other plants growing near *Cycas* or

Azolla showed a high level of nitrate reductase activity even without pre-incubation in KNO$_3$.
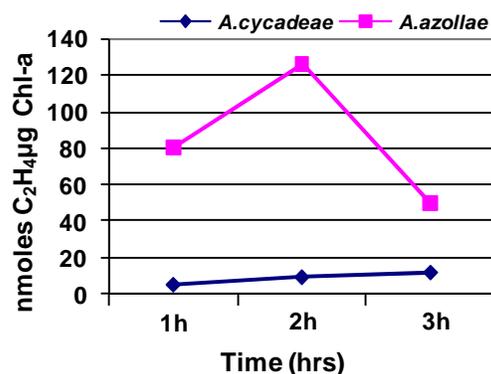

Fig 1. Nitrogenase Activity of Laboratory grown cultures of *Anabaena cycadeae* and *Anabaena azollae*
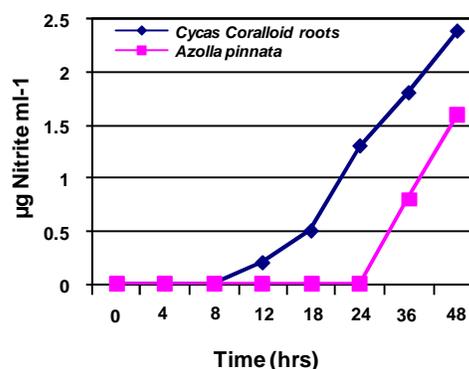

Fig 2. Induction of Nitrate Reductase in *Cycas* coralloid roots and *Azolla* plants

This suggests that a symbiotic forms; fixed nitrogen is supplied by an endophyte to the host, as such the host does not depend on any inorganic nitrogen sources. It is also possible that N_2 fixing symbiotic higher plant need a higher concentration of NO_3^- for the induction of nitrate reductase, which in reality is never available in the natural conditions where they grow.

From the current results it was evident that apparent differences exist in the N_2 fixing capacity of various symbiotic systems. In the symbiotic systems investigated the nitrogenase activity exhibited positive correlation with the heterocyst frequency, and lastly the combined nitrogen sources present in the natural systems were in no way inhibitory of active nitrogen fixation by *Azolla* and *Cycas*.

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2/23/2015