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Nitrogenase Activity and Iron-Superoxide Dismutase Localization in *Cycas revoluta* Thunb. cyanobionts

MARIA GRILLI CAIOLA and ANTONELLA CANINI
Dipartimento di Biologia, Università di Roma 'Tor Vergata', Via della Ricerca Scientifica, I-00133, Roma (Italy)
Tel. 0039-6-72594344, Fax 0039-6-2023500

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Abstract

Heterocyst frequency, nitrogenase activity and iron-superoxide dismutase (Fe-SOD) were detected in the cyanobiont zone of coralloid roots. Heterocyst frequency of cyanobionts increased from the growing tips (20%) of coralloid roots towards the median segments. Degenerative heterocysts appeared in the median segments and its percentage increased toward the basal ones (60%). Nitrogenase activity was highest in the apical segments (1.1 nmol $C_2H_4 \cdot \mu g\ Chl^{-1} \cdot h^{-1}$) and decreased in the median segments, reaching a minimum level in the basal ones (0.2 nmol $C_2H_4 \cdot \mu g\ Chl^{-1} \cdot h^{-1}$). Fe-SOD was localized both in vegetative cells and in heterocysts of the cyanobionts. Fe-SOD gold particle density in vegetative cells (28–35 gold particles/μm^2) did not vary along the coralloid axis, whereas heterocysts contained more Fe-SOD gold particles than vegetative cells, mainly in the apical zone (50 gold particles/μm^2). In heterocysts of the base, Fe-SOD gold particles were about 50% less than those of the apex, whereas only a few gold particles were detected in degenerated heterocysts. Therefore, in heterocysts of the cyanobiont of *Cycas revoluta*, Fe-SOD could represent a general protection of cells against damaging superoxide radicals generated in the cyanobiont zones with high oxygen concentration.

Keywords: cyanobiont; heterocysts, iron-superoxide dismutase (immunogold labelling)

Abbreviations: Fe-SOD = iron-superoxide dismutase; ARA = nitrogenase activity

1. Introduction

Cycas revoluta Thunb. carries apogeotropic coralloid roots, containing a zone of heterocystous, nitrogen fixing cyanobionts. This zone is constituted of peridermal, radially elongated cells forming intercellular spaces filled with mucilage in which cyanobacteria are located. Previous studies on cycad coralloid root symbionts indicate that heterocyst frequency increases from the apex to the base of the coralloid tissue (Grilli Caiola, 1980; Lindblad and Bergman, 1986; Lindblad et al., 1985), whereas acetylene reduction activity is greatest at the growing tip and gradually declines toward the base (Lindblad et al., 1985; Lindblad et al., 1991). In addition, although cyanobionts possess phycobiliproteins and carboxysomes, they do not carry out oxygenic photosynthesis or CO_2 fixation and data on isolates from *Macrozamia* suggest that the cyanobacteria within the coralloid roots are differentiated specifically for functioning in a microaerobic environment (Lindblad et al., 1991). A recent study carried out with microelectrodes (Canini and Grilli Caiola, 1993) on the ion composition of *Cycas revoluta* coralloid roots indicates that in the cyanobiont zone, oxygen pressure is almost in equilibrium with the atmosphere and does not change along the coralloid axis. These data suggest the existence in cycad coralloid roots of an efficient mechanism to protect nitrogenase from the inhibiting effects of oxygen.

A possible nitrogenase protection mechanism could be performed by the action of Fe-SOD. SOD has been localized in *Azolla* symbionts (Canini et al., 1992a), in nitrogen fixing, free-living *Anabaena cylindrica* (Canini et al., 1992b) and in *Nostoc* sp. isolated from cycad coralloid roots and grown in culture (Canini et al., 1992c). In both these cyanobacteria, the amount of Fe-SOD in the heterocysts was higher compared to that of vegetative cells and akinetes. Moreover, more enzyme was detected in culture-grown cells at higher light intensities, indicating a role for Fe-SOD against superoxide radicals originating both from respiration and photosynthesis. In this perspective, cycad coralloid roots appear to be a good model for studying the role of Fe-SOD as a protection mechanism for nitrogenase activity against oxygen radicals damage since the cyanobiont grows in the dark and the cyanobiont zone does not contain any photosynthetic oxygen-evolving cells.

In this paper we compare Fe-SOD distribution in relation to heterocyst frequency and acetylene reduction activity of the cyanobionts living in different segments of *Cycas revoluta* coralloid roots.

2. Materials and Methods

Plant

Coralloid roots were collected from plants of *Cycas revoluta* Thunb. growing in the Botanical Garden of the University of Naples (Italy). Freshly collected coralloids were sliced in four parts starting from the tip as follows: (1) apical segment, from the tip to 2 mm; (2) subapical segment, from 2 to 4 mm; (3) median segment, from 4 to 6 mm; (4) basal segment, from the previous to the basis of coralloid. Each segment was used for the following determinations.

Heterocyst frequency (HF)

Samples of each segment were squeezed between a slide and a slide cover and the percentage of heterocyst was calculated according to Grilli Caiola (1980). In basal segments heterocyst frequency was calculated as the percentage of healthy and degenerated heterocysts. Heterocysts were considered to be degenerated when they showed an irregular cell wall and envelope with scarce or collapsed cytoplasm.

Acetylene reduction activity

Acetylene reduction activity was detected according to Hardy et al. (1968) with some modifications. Approximately 40 sections of each coralloid root segment were put in 14 ml vials containing 1 ml of distilled water. The vials were incubated in the dark, for 6 hr, at 25° C, under 90% air–10% acetylene. After 2 hr, 1 ml aliquots of the gas phase were removed, and the ethylene formed was determined by gas chromatography. Nitrogenase activity values are reported as nmol ethylene produced per μg Chl per hr.

Chlorophyll content

Sections of coralloid roots were disrupted by using a potter and filtered. Chl content was determined by extracting pigments of cyanobacterial cells in methanol and measuring the absorbance at 665 nm (Mckinney, 1941).

Protein determinations

Protein concentrations were determined according to Lowry et al. (1951) using bovine serum albumin as standard.

Western blotting and immunoprecipitation

Coralloid roots after disruption in a mortar were suspended in phosphate-buffered saline (PBS; 20 mM Na-K phosphate buffer, pH 7.4, containing 150 mM NaCl), and the suspension was filtered on nylon mesh 20 μm large. Filaments of the cyanobiont were separated from the filtrate by using Percoll gradients at density 1.080 g/ml containing 2.5 M sucrose. After a 50 min centrifugation at 70,000 g, the band containing cyanobiont filaments was removed and washed two times with PBS. Filaments were sonicated in an ice-bath at 18 W for 5 min at 30 s intervals. The progress of cell disruption during the sonication steps was monitored using a light microscope. Cell debris was removed by centrifugation at 26,000 g for 30 min. The supernatant (40 μg total protein) was heated at 100° C for 10 min, in a Multiblok heater (Lab-line Instrument: Melrose Park, IL, USA). After polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate and β-mercaptoethanol, the separated bands were electroblotted onto nitrocellulose membranes using a BioRad Transblot Cell (BioRad Laboratories, Richmond, Cal., USA) (Towbin et al., 1979). Immunoassay of electroblotted nitrocellulose was carried out as described in Canini et al. (1992b). The primary antibody was a rabbit anti-Fe-SOD from *Anabaena cylindrica* (Canini et al., 1992b) diluted 1:300 (v/v). Controls were performed by omitting the primary antibody and by staining the gels after the transfer of proteins onto the nitrocellulose membranes. The Fe-SOD from *A. cylindrica* was used as a known antigen.

Immunogold labelling and transmission electron microscopy

Sections of each group of coralloid segments were fixed for 3 hr in a 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2. The sections were dehydrated in a graded ethanol series and embedded in Epon 812 resin (Agar Aids Stanstead, Essex, UK). Immunogold labelling of Fe-SOD was carried out on ultrathin sections obtained with a diamond knife as described in Canini et al. (1992b). Anti-Fe-SOD antibody was used at 1:500 dilution and the secondary antibody at 1:25 dilution. Gold particles of 10 nm conjugated with the secondary antibody were used. In control samples, the primary antibody was omitted. Some grids, after immunogold labelling, were also post-stained with uranyl acetate and lead citrate (Reynolds, 1963). Electron microscopical observations and micrographs were made by using a CEM Zeiss electron microscope operating at 80 kV. Counting of the gold particle density/μm^2 area of the cells was carried out on at least 8 electronmicrographs of each cell type and from each group of segments obtained at the same magnification.

3. Results

Heterocyst frequency and acetylene reduction activity (ARA)

Heterocyst frequency in different segments of coralloid roots are shown in Fig. 1. The total number of heterocysts increased from 20% at the tip to 60%

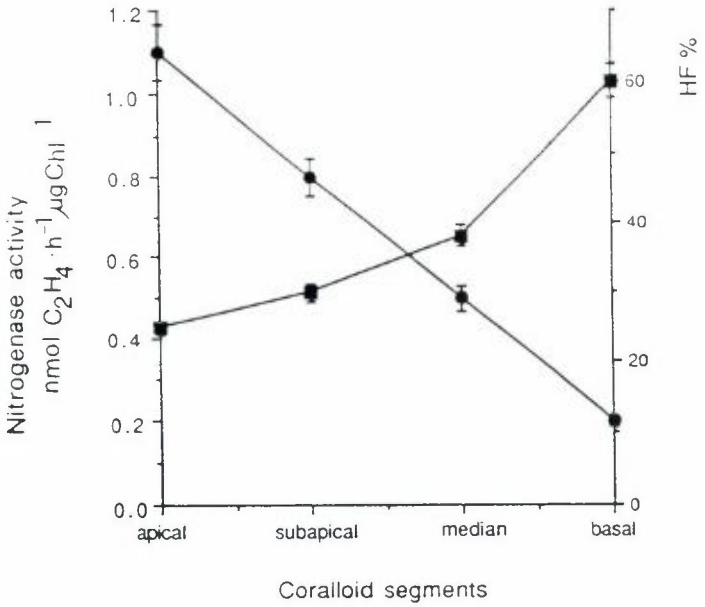

Figure 1. Heterocyst frequency (HF) (■) and nitrogenase activity activity (ARA) (●) of *Cycas* cyanobionts in the various segments of coralloid roots. The values are the average of three separate experiments ± SD.

at the base, and heterocyst's morphology changed. At the tip, heterocysts appeared to be healthy, but starting from subapical segments, the percentage of degenerated heterocysts increased to 24%. Acetylene reduction activity decreased gradually from the apex with 1.1 nmol $C_2H_4 \cdot \mu g\ Chl^{-1} \cdot h^{-1}$ to 0.2 nmol $C_2H_4 \cdot \mu g\ Chl^{-1} \cdot h^{-1}$ at the basal segments of the coralloids (Fig. 1).

Labelling of Fe-SOD

The specificity of the polyclonal rabbit-anti Fe-SOD antibody was examined by Western blotting and immunoprecipitation (Fig. 2). In the cyanobiont extract, only a band at 21 KDa reacted with the Fe-SOD antibody from *Anabaena cylindrica* (Canini et al., 1992b). The two minor bands at 35–38 KDa present in the purified *Anabaena cylindrica* were also missing in the *Cycas*

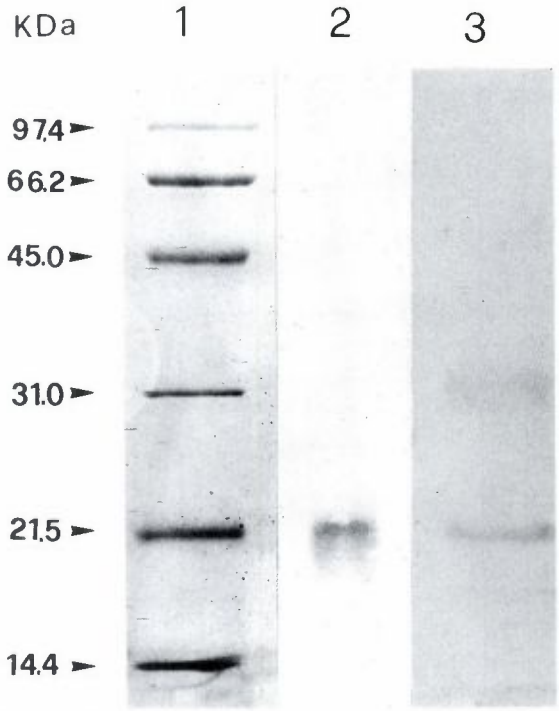

Figure 2. Immunoblotting of cell extracts of *Cycas* cyanobiont after SDS-PAGE with purified Fe-SOD from *Anabaena cylindrica* (Lane 2); molecular-weight markers: 97000, phosphorylase; 66000, bovine serum albumin; 45000 ovalbumin; 31000 carbonic anhydrase; 21500, soybean trypsin inhibitor; 14400 lysozyme (Lane 1); purified Fe-SOD from *Anabaena cylindrica* after SDS-PAGE (Lane 3).

cyanobiont when larger amounts of protein were loaded on the gel. In Fig. 2 the purified Fe-SOD from *A. cylindrica* is shown as a known antigen.

The cyanobiont zones contained vegetative cells and heterocysts. Akinetes were never observed. Fe-SOD labelling appeared in all cyanobiont cells of all segments, however the distribution of the enzyme differed. In all the observed sections, Fe-SOD was localized both in vegetative cells (Fig. 3) and in heterocysts (Fig. 4), but we did not observe remarkable differences from the tip to the base among the vegetative cells. Differences were recorded between vegetative cells and heterocysts. The latter contained about 1.5 times the amount of Fe-SOD as was present in the vegetative cells. In addition, heterocysts were richer in Fe-SOD in the apical segments than in the basal segments. In the basal segments, heterocysts contained about 50% less gold particles than apical ones (Fig. 5), whereas degenerated heterocysts contained even less Fe-SOD (Fig. 6). The Fe-SOD labelling appeared to be mainly concentrated in the nucleoplasm of the vegetative cells while it was homogeneously distributed in the cytoplasm of the heterocysts. The density of

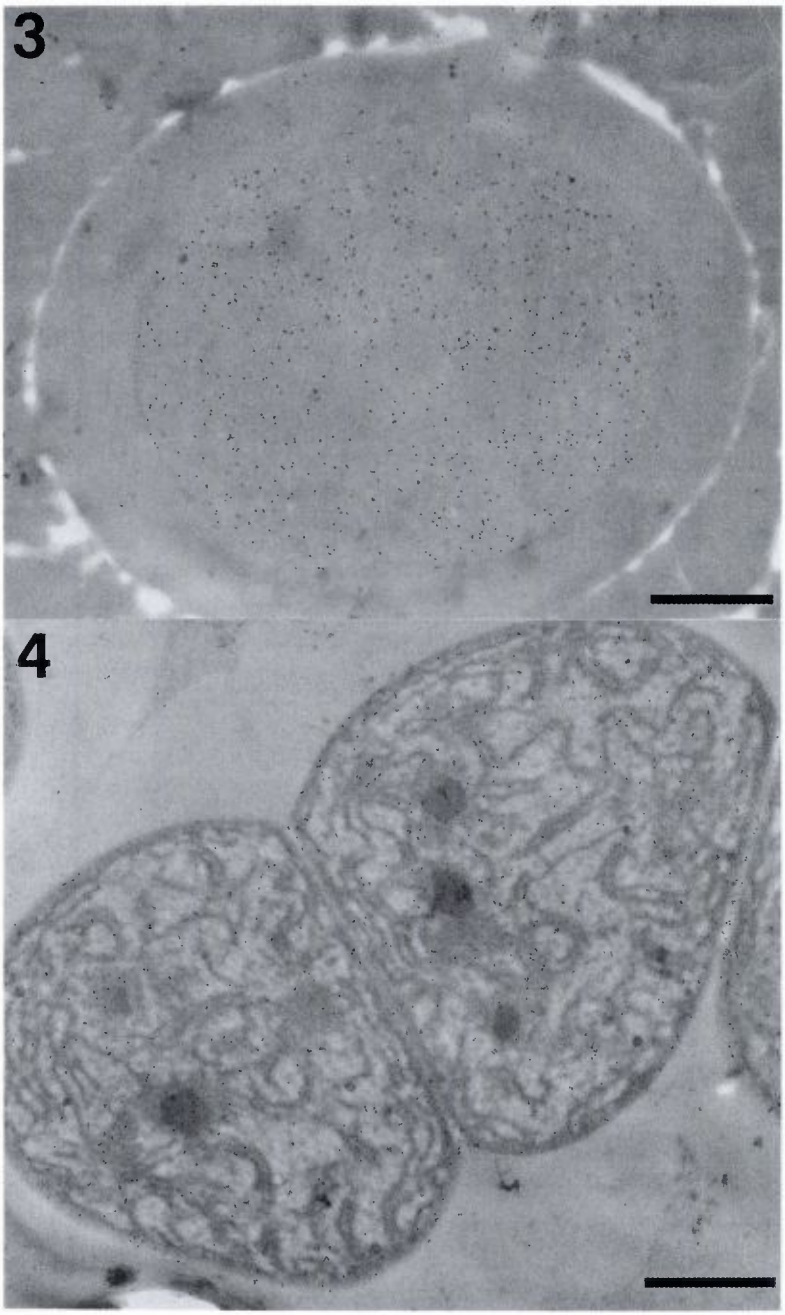

Figure 3. Immunogold labelling of Fe-SOD in heterocyst of *Cycas* cyanobiont located in the apical segments of coralloid root.

Figure 4. Immunogold labelling of Fe-SOD in vegetative cell of *Cycas* cyanobiont in the apical segments.

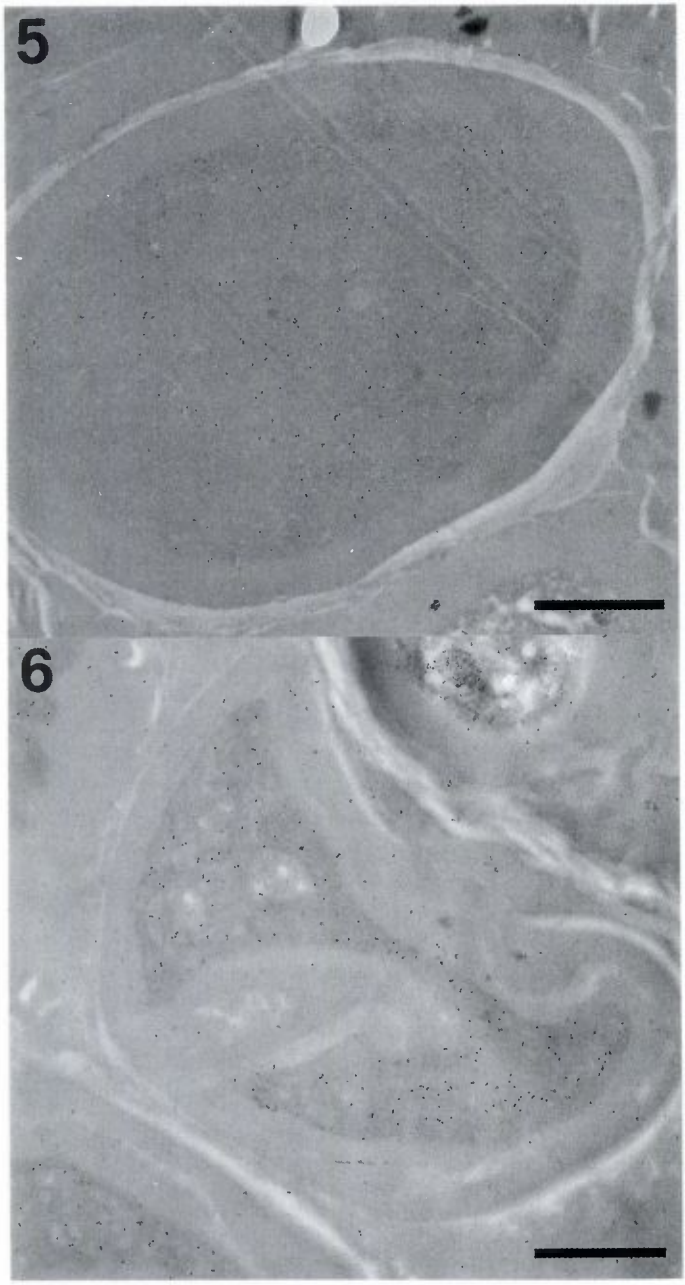

Figure 5. Localization of Fe-SOD in a healthy heterocyst of *Cycas* cyanobiont at the base of coralloid root.

Figure 6. Immunogold labelling of Fe-SOD in degenerated heterocysts of cyanobiont at the base of coralloid root.

gold particles of heterocysts and vegetative cells of different coralloid segments are reported in Table 1.

4. Discussion

In the coralloid roots of *Cycas revoluta*, variations of Fe-SOD content appear related to nitrogenase activity and cyanobiont cell types as demonstrated by the higher gold particle density in heterocysts of apical and median segments. In contrast, oxygen concentration did not vary significantly from the apical to the basal segment (Canini and Grilli Caiola, 1993). The immunogold labelling of Fe-SOD indicated that the enzyme is more abundant in heterocysts than in vegetative cells and that Fe-SOD is localized mainly in the heterocysts of apical segments where ARA is highest. Since photosynthetic oxygen evolution by the cyanobiont of the coralloid roots is very low (Lindblad et al., 1991; Perraju et al., 1986), our hypothesis is that respiration, which supplies nitrogenase with ATP and electrons, must be the major process generating superoxide radicals. The presence of Fe-SOD could provide a protection against the oxygen-radicals which may react directly with nitrogenase or initiate a cascade of destructive events and cause inhibition of the enzyme. This protection mechanism appears to be very efficient within the heterocysts if we consider the high oxygen concentration to which these cells are subjected. Fe-SOD has already been detected in *Nostoc* from *Cycas revoluta* coralloid roots isolated and grown in culture (Canini et al., 1992c).

The variation of ARA in *Cycas revoluta* coralloid roots appears to agree with those found in the same cycad species by Lindblad and Bergman (1986), although we found lower nitrogenase activity values.

In degenerative cells, Fe-SOD was present in lower amounts than in active heterocysts, indicating the presence of an oxidative metabolism not strictly related to nitrogen fixation. A possible role of Fe-SOD in cyanobionts of basal segments could be that of defending the cells against the oxygen radicals

Table 1. Density of gold particles (10 nm) due to Fe-SOD labelling in heterocysts and vegetative cells of apical, subapical, median and basal coralloid root segments. The data are means ± SD of three independent counts on at least 8 electronmicrographs.

Segments	Vegetative cells	Heterocysts
	Gold particles density/μm^2 cell area)	
Apical	35±2	50±3
Subapical	30±4	40±2
Median	28±2	35±5
Basal	30±4	20±4

present during cyanobiont senescence or host attack. So far, nothing is known about the host effects on cyanobionts. Degeneration of cyanobiont cells increases from median segments to the base of coralloid roots. In these parts we noted a remarkable amount of Fe-SOD in the heterocysts but ARA was reduced to a minimal level while the ammonium concentrations were high (Canini and Grilli Caiola, 1993). In this case we cannot identify the effect of the enzyme as protecting only nitrogenase but there is probably a general cell protection against superoxide radicals originating from an oxidative metabolism both in active and degenerative heterocysts as well as in vegetative cells.

The origin of degenerative cells is unknown at present. Lindblad et al. (1991) attributed morphology modifications of cyanobionts to microaerobic conditions present in coralloid roots of *Macrozamia*. A possible cause of cyanobiont degeneration could be the variation in composition of the mucilage filling the intercellular spaces in which the cyanobionts reside and where the accumulation of ammonium and calcium (Canini and Grilli Caiola, 1993), and variation in the mucilage polysaccharide components have been reported (Grilli and Trabucchi, 1963; Grilli Caiola, 1980).

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