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Reconstitution of a cycad-cyanobacterial association

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ABSTRACT *Zamia furfuracea* and its cyanobiont *Nostoc* FUR 94201 were separated and reunited in the laboratory to reconstitute a functional symbiosis between a cycad and cyanobacterium. Reconstitution was achieved also with *Nostoc* 2S9B, a soil cyanobacterium. The identities of the cyanobacteria were confirmed by amplified fragment length polymorphisms, using the highly polymorphic intergenic region separating the 16S RNA and 23S RNA genes. Scanning electron microscopy indicated the presence of mucilaginous material on the surface of roots present when *Nostoc* FUR 94201 was cocultivated with the plant.

Keywords: cyanobacteria -- mucilage -- *Nostoc* -- symbiosis -- *Zamia*

1. Introduction

Cycads, ancient plants endemic to the tropics and subtropics, are the only known gymnosperms that form symbiotic relationships with cyanobacteria (or any other bacterium). Cycads harbor their symbiotic partner within specialized coralloid roots, where the cyanobacteria provide fixed nitrogen, possibly in exchange for photosynthate (Lindblad and Bergman, 1990).

Cyanobacteria have a remarkably broad host range and thus differ from rhizobia, whose host-range is almost exclusively confined to legumes, and from *Frankia*, which infects only angiosperms. Cyanobacteria are able to form symbiotic associations with genera representative of four plant lineages: an angiosperm, *Gunnera* (Bergman et al., 1992); several bryophytes, e.g., *Anthoceros* (Meeks, 1990); a fern, *Azolla* (Braun-Howland and Nierzwicki-Bauer, 1990), and cycads (Lindblad and Bergman, 1990). Reconstitution of the association between purified cyanobacteria and two very different plant hosts, *Gunnera* and *Anthoceros*, has led to the surprising result that most symbiotic cyanobacteria are infective regardless of their original host (Enderlin and Meeks, 1983; Johansson and Bergman, 1994). It would be interesting to test the host range of symbiotic cyanobacteria from diverse sources on axenic cycads as well, but there has been no report of successful infection of a cycad with even a cyanobacterium native to the plant. We sought, therefore, to demonstrate the reestablishment of a cycad-cyanobacterial symbiosis.

2. Materials and Methods

Cyanobacterial strains used in this study were *Nostoc* FUR 94210 (isolated from *Zamia furfuracea* as described below), *Nostoc* 2S9B (Gantar et al., 1991; isolated from Yugoslavian soil and capable of infecting wheat roots), *Nostoc* PCC 73102 (isolated from *Macrozamia*), and *Nostoc* ATCC 29133 (ostensibly the same isolate as PCC 73102 but with different laboratory histories and morphologies). The latter two strains were gifts from Jack Meeks (University of California at Davis). DNA from these strains was isolated as previously described (Cai and Wolk, 1990), and DNA from other strains was generously provided by Karin Forslund and Peter Lindblad (Uppsala University). Strains were grown shaken at 30°C in BG11 liquid medium (Rippka et al., 1979), modified as previously described (Elhai and Wolk, 1990), or in $BG11_0$, identical to BG11 except that $NaNO_3$ is replaced by an equimolar concentration of NaCl. BG11 agar plates consisted of BG11 medium supplemented with separately autoclaved Bacto-agar (Difco Laboratories) to a final concentration of 1.4%.

To isolate endogenous cyanobacteria from the cycad *Zamia furfuracea*, a coralloid root a few centimeters below the surface of the soil was taken from *Z. furfuracea* seedling, grown in a pot from a seed obtained from Fairchild Tropical Garden. The root was rinsed with water to remove excess soil and externally sterilized by swirling for 5 minutes in a mixture of triton X-100 and 10% commercial bleach. The root was washed three times in sterile water, once with 100% ethanol, and three more times with sterile water. Using a sterile razor, the root was sliced across, making visible the green ring of the cyanobacterial zone. Cyanobacteria were scooped out from inside of the coralloid root with a sterile toothpick and streaked onto a BG11 plate.

Axenic *Z. furfuracea* were grown for reconstitution experiments from seeds (obtained from Fairchild

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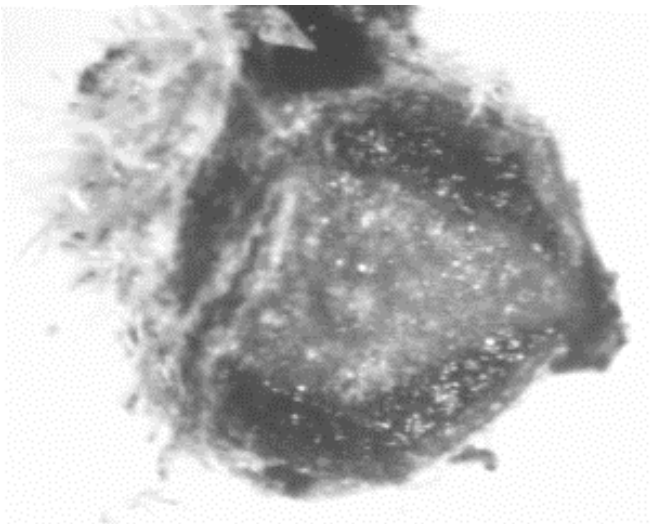

Fig. 1. Cross section of a coralloid root of *Zamia furfuracea*. The dark green region consists of densely packed cyanobacteria. The root had a width of roughly 3 mm.

Tropical Garden) that were surface-sterilized as described above. The seeds were allowed to germinate at 30° in an axenic container with sand and perlite and seedlings were grown under continuous light, watered routinely with sterile water and about once a month with BG11 medium. Six months after germination, the seedlings were inoculated with 1 ml of a dense cyanobacterial culture. At least three seedlings per cyanobacterial strain were inoculated. Two months after inoculation, coralloid roots from the seedlings were harvested, sliced, and observed for the presence of a green cyanobacterial zone.

Samples of infected and non-infected coralloid roots were prepared for scanning electron microscopy by fixation in 3% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, for 48 h. Fixed roots were then dehydrated in a series of ethanol solutions (50-100%) and critical point dried. Dried samples were mounted and coated with gold-palladium prior to observation under an ISI scanning electron microscope.

Cyanobacterial DNA was amplified using the polymerase chain reaction (PCR) directed by the following two primers. One primer, 5'-tggatcCTAGT AAT(TC)GC(ATC)(GA)GTCAG-3', was chosen to match precisely a region conserved amongst 16S rDNA sequences (graciously provided by Seán Turner, Louisiana State University) of 10 heterocystous cyanobacteria. The primer also matches the corresponding sequences from most chloroplasts and some heterotrophic bacteria (Dams et al., 1988). The capitalized sequence corresponds to bases 1289 to 1306 of one copy of 16S rDNA from *Anabaena* PCC 7120 (Ligon et al., 1991; EMBL accession no. X59559). The second primer, 5'-actcgAG(CG)(AT)ATCC(AC)(CG)C (GA)(TC)AAGCC-3', was chosen to match a sequence at the 5' end of the 23S rDNA gene sequences highly conserved amongst chloroplasts and cyanobacteria (Gutell and Fox, 1988). The capitalized sequence corresponds to bases 33 to 16 of both copies of 23S rDNA from *Synechococcus* PCC 6301 (Kumano et al., 1983; Douglas and Doolittle, 1984). Lower case letters indicate nonmatching bases added to provide BamHI and XhoI restriction sites, respectively. The PCR reaction was catalyzed by AmpliTaq DNA polymerase, Stoffel Fragment (Perkin Elmer), in a buffer containing 4 mM $MgCl_2$, 250 µM NTPs, 0.5 µM of each primer, and 40 ng cyanobacterial DNA or a small volume of cyanobacteria (Wilmotte et al., 1993), in a total volume of 50 µl. The reaction followed the protocol: 94°C 15 sec, 40°C 1 min, 55°C 3 min, 72°C 1 min, for four cycles; 94°C 15 sec, 55°C 1 min, 72°C 1 min, for thirty-six cycles; 70°C 10 min. Amplified fragments were by electrophoresis separated on a 2.5% agarose gel.

3. Results

A cyanobacterial strain was cultured from the green cyanobacterial zone (Fig. 1) of a coralloid root of *Z. furfuracea*. The cyanobacterium was filamentous, elaborated heterocysts (specialized nitrogen-fixing cells) when deprived of nitrogen in the medium, and

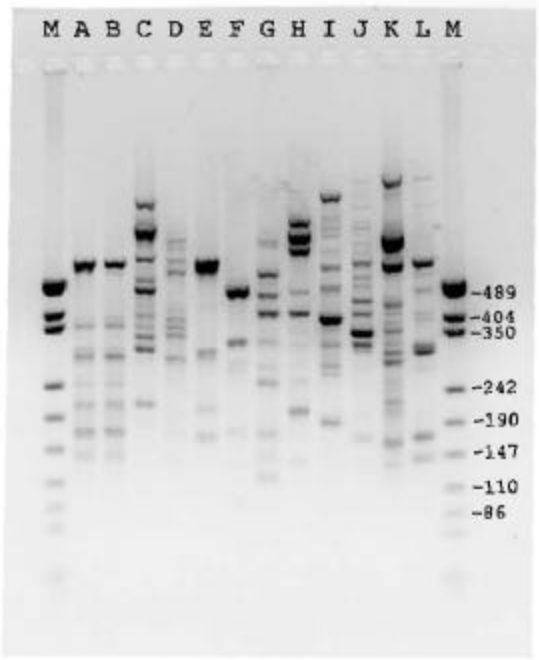

Figure 2. Agarose gel electrophoresis of DNA fragments amplified by primers flanking the 16S rDNA-23S rDNA internal transcribed spacer. Lanes are: (M) size markers from pUC12 digested with *Hpa*II shown with sizes in base pairs, (A) *Nostoc* FUR 94210, (B) cyanobacterium isolated from *Z. furfuracea* infected with *Nostoc* FUR 94210, (C) *N. gunnera*, (D) *N. encephalartos*, (E) *N. zamia*, (F) *Nostoc* 2S9B, (G) *Nostoc* ATCC 7422, (H) *Nostoc* ATCC 8005, (I) *Nostoc* PCC 73012, (J) *Anabaena azolla*, (K) *Synechocystis* PCC 6803, (L) *Synechococcus* PCC 7942.

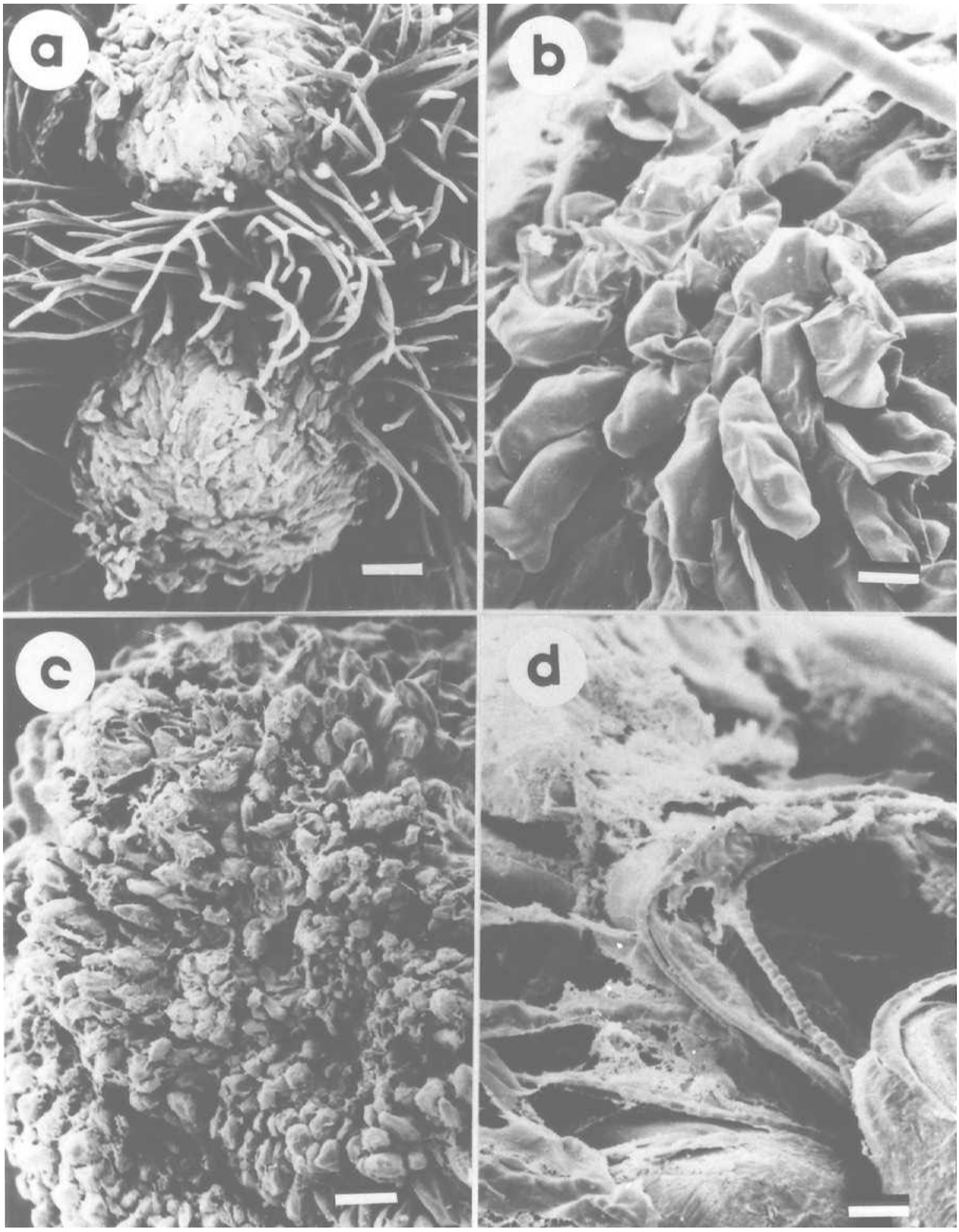

Figure 3. Scanning electron micrographs of rootcaps of axenically grown *Zamia furfuracea* (**a** and **b**) the same species after infection with *Nostoc* FUR 94210 (**c** and **d**). Bar markers represent 200 μm, 30 μm, 200 μm, and 20 μm for **a** through **d**, respectively.

differentiated into motile hormogonia in response to water extract from macerated seeds of *Zamia furfuracea*. On the basis of these and other characteristics (Rippka, 1988), the strain was named *Nostoc* FUR 94210.

Reconstitution of the plant with its native cyanobacterium and with other cyanobacteria was attempted using seedlings of *Z. furfuracea*. Cyanobacterial growth was clearly evident and growth of the cycad was sustained in all inoculated containers but not in the uninoculated control. Two months after inoculation, several coralloid roots were sectioned. All coralloid roots taken from a plant grown with *Nostoc* FUR 94210 exhibited green cyanobacterial zones typical of infected cycad roots, as did coralloid roots from three plants inoculated with *Nostoc* 2S9B. In contrast, coralloid roots taken from plants grown with *Nostoc* sp. PCC 73102, *Nostoc* sp. ATCC 29133, or no strain at all did not exhibit green cyanobacterial zones.

The cyanobacteria isolated from the coralloid roots of inoculated seedlings were to all appearances identical to the strains with which they had been inoculated. To confirm this identity, we used primers directed to conserved sequences within 16S rRNA and 23S rRNA to amplify the region between the two genes. This region is known to be highly polymorphic amongst cyanobacteria isolated from cycads (Forslund, Martel, Elhai, and Lindblad, unpublished observations), and in fact the resulting banding pattern easily distinguishes *Nostoc* FUR 94210, *Nostoc* 2S9, and *Nostoc* PCC 73102 from several other *Nostocs* and from each other (Fig. 2). As expected, the banding patterns of fragments amplified from DNA of the recovered cyanobacteria were identical to that of fragments amplified from DNA of the inoculants. One strain, *Nostoc zamia*, isolated had a pattern similar though not identical to that of *Nostoc* FUR 94210, the only other cyanobacterium tested that was isolated from *Zamia*. The identification of a recovered cyanobacterium as *Nostoc* 2S9B (not shown) excludes the possibility that cyanobacterial growth arose from incomplete sterilization of the seed.

Cycads developed coralloid roots whether or not they were grown axenically or in the presence of cyanobacteria, in accordance with previous results (Lamont and Ryan, 1977; Webb, 1982). Coralloid roots from infected and uninfected cycads were compared by scanning electron microscopy in order to assess what effect the presence of *Nostoc* FUR 94210 had on the plant. The surface of coralloid roots of uninfected plants had loosely packed cells (Figs. 3a and 3b). The surface of coralloid roots from plants grown in the presence of *Nostoc* FUR 94210 was little different, except for a pronounced layer of mucilaginous material (Figs. 3c and 3d). This material was also observed on coralloid roots of plants infected with other strains. Short cyanobacterial filaments, presumably hormogonia, could be observed within the mucilaginous matrix, sometimes between the spaces separating the cells of the root.

Discussion

The results presented here demonstrate the possibility of reconstituting a cycad-*Nostoc* association in the laboratory. *Zamia* thus join *Anthoceros* (Enderlin and Meeks, 1983) and *Gunnera* (Johansson and Bergman, 1994) as plants in which the requirements for cyanobacterial associations may be studied. Just as *Anthoceros* and *Gunnera* form associations with a wide variety of different *Nostoc* strains, *Zamia* is able to accept both native cyanobacteria and some cyanobacteria foreign to the genus.

Controversy has long surrounded attempts to isolate a cyanobacterial symbiont associated with *Azolla*. It is now evident that strains that were readily cultured from *Azolla* associations were in fact epiphytes unrelated to the major symbiont (Gebhardt and Nierzwicki-Bauer, 1991), which grows very poorly in culture (Tang et al., 1990). Attempts to culture symbiotic cyanobacteria from lichen have suffered from similar uncertainty (Kardish et al, 1990). There are no such complications with the *Zamia-Nostoc* association. The identity between the PCR fingerprints of the cultured and endogenous cyanobacteria (Fig. 2) makes it very likely that the isolated *Nostoc* FUR 94210 is the true symbiont of *Zamia*.

The 16S-23S rDNA intergenic region used in this study to confirm the identify of the *Nostoc* symbionts has proven to be highly polymorphic among closely related heterotrophic bacteria (Jensen and Straus, 1993), among cyanobacteria in general (Wilmotte, 1994), among cyanobacteria isolated from hornworts (West and Adams, 1997), among cyanobacteria isolated from cycads (Forslund, Martel, Elhai, and Lindblad, unpublished observations), and from symbiotic *Nostoc* (this study). On the other hand, Neilan et al. (1997) found that a DNA fragment amplified from the intergenic region of a strain of *Nostoc* had the same length as that amplified from cyanobacteria of two closely related genera (*Anabaena* and *Aphanizomenon*). While this result does not directly conflict with our own, it is nonetheless surprising. The complex banding patterns observed in Fig. 2 are expected, considering the multiple copies of the genes in cyanobacteria (Ligon et al, 1991; Chen and Widger, 1993; Churin et al, 1995) and the propensity of the different PCR products from this region to combine to form heteroduplexes (Jensen and Straus, 1993).

Observations on the infection of previously axenic roots (Fig. 3d) provide graphic illustration of the idea supported by Milindasuta (1975) that cyanobacteria enter uninfected coralloid roots through spaces between cells of the rootcap, which at low frequency lead to

breaks in the dermal layer and cortical channels beyond. Staff and coworkers differ somewhat from this view in asserting that entry occurs through breaks distal from the rootcap (Nathanielsz and Staff, 1975; Staff and Ahern, 1993). The choice of plant may account for these differences (*Macrozamia* by Staff, *Zamia* by Milindasuta and us), but it is perilous to draw firm conclusions on the basis of so few observations.

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