In vitro colonization and increase in nitrogen fixation of seedling roots of black mangrove inoculated by a filamentous cyanobacteria

Gerardo Toledo, Yoav Bashan, and Al Soeldner

Abstract: An isolate of the filamentous cyanobacterium *Microcoleus* sp. was obtained from black mangrove aerial root (pneumatophore) and inoculated onto young mangrove seedlings to evaluate N_2-fixation and root-colonization capacities of the bacterium under in vitro conditions in closed-system experiments. N_2 fixation (acetylene reduction) gradually increased with time and reached its peak 5 days after inoculation. Later, it decreased sharply. The level of N_2 fixation in the presence of the plant was significantly higher than the amount of nitrogen fixed by a similar quantity of cyanobacteria on a N-free growth medium. The main feature of this root colonization was the gradual production of a biofilm in which the cyanobacterial filaments were embedded. Visible biofilm production increased with time until it completely covered the entire root system of the plant. The in-and-out movement of cyanobacterial filaments from the biofilm probably allows colonization of uncolonized portions of the root several days after the initial inoculation. This is, to the best of our knowledge, the first report of the artificial inoculation of cyanobacteria on marine mangroves.

Key words: *Avicennia germinans*, beneficial bacteria, biofilm, black mangrove, diazotrophic cyanobacteria, *Microcoleus* sp., nitrogen fixation, plant growth-promoting bacteria, root colonization.

Resume : Un isolat de la cyanobactérie filamenteuse *Microcoleus* sp. a été obtenu de racine aérienne (pneumatophore) du manguier noir et inoculé à de jeunes pousses de manguier pour évaluer la fixation d'azote et la capacité de la bactéria à coloniser les racines dans des conditions in vitro et expériences en système fermé. La fixation d'azote (reduction de l'acétylène) a augmenté graduellement avec le temps et atteint son pic 5 jours après l'inoculation; ultérieurement, elle a décrû rapidement. Le niveau de fixation de l'azote en présence d'une plante a été significativement plus élevé que la quantité d'azote fixée par une quantité similaire de cyano-bactéries croissant sur un milieu libre d'azote. Le trait principal de cette colonisation des racines a été la production graduelle d'un biofilm dans lequel étaient enfouis les filaments cyanobactériens. La production d'un biofilm visible s'est accrue avec le temps jusqu'à complet recouvrement de tout le système racinaire d'une même plante. Les mouvements d'entrée et de sortie du biofilm des filaments cyanobactériens permettent probablement la colonisation des portions non colonisées des racines, plusieurs jours après l'inoculation de départ. L'inoculation artificielle de cyanobactéries sur les manguiers marins est rapportée, à notre connaissance, pour la première fois.

Mots clés : *Avicennia germinans*, bactéries bénéfiques, biofilm, manguier noir, cyanobactéries diazotrophes, *Microcoleus* sp., fixation d'azote, bactéries promotrices de croissance végétale, colonisation des racines. [Traduit par la rédaction]

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Introduction

The inoculation of crop and forest plants with associative beneficial bacteria has generated well-documented techniques that have recently reached their experimental peak en route to commercialization (Okon and Labandera-Gonzalez 1994; Tang 1994; Paau et al. 1991). The most commonly used organisms belong to the biocontrol group of pseudomonads (Kloepper et al. 1989), *Azospirillum* (Bashan and Levanony 1990), *Klebsiella* (Haahtela et al. 1986), *Azotobacter* (Pandey et al. 1989), and *Bacillus* (Berge et al. 1990; Holl and Chanway 1992). The study of cyanobacterial inoculation of plants has been concentrated on rice (Ghosh and Saha 1993; Melting et al. 1988; Roger et al. 1987; Singh and Singh 1989; Yanni 1992) and recent reports on the inoculation of wheat plants (Gantar et al. 1991*a*, 1991*b*, 1993).

Mangrove forests are essential for sustainable coastal fisheries. They supply large amounts of carbon and nitrogen to the environment and act as a major refuge and feeding ground for many economically important fish and shellfish (Bashan and Holguin 1995; Robertson and Blaber 1992). Once a mangrove forest has been cleared, the coastal fishing industry may be irreversibly decimated (Por 1984).

Diazotrophic activity in mangroves and in other marine plants has been observed throughout the world (Holguin et al. 1992; Potts 1984; van der Valk and Attiwill 1984; Zuberer 1984; Zuberer and Silver 1978) but usually without any reference to the particular species of microorganisms, which vary from site to site. Marine cyanobacterial populations are an integral and a major component of the microbiota in every mangrove system (Hussain and Khoja 1993; Potts 1979, 1980; Potts and Whitton 1980). They colonize any submerged surface such as sediment, roots, aerial roots, branches, and trunks of mangrove (Hicks and Silvester 1985; Sheridan 1991; Zuberer and Silver 1978). Yet, the exact interaction between cyanobacteria and mangroves or any resulting mutual benefits have yet to be established.

All cyanobacteria adhering to surfaces share two properties: (*i*) they have a hydrophobic cell surface and (*ii*) they form biofilms. During the colonization process, the membrane changes from hydrophilic to hydrophobic, thereby enhancing the attachment (Shilo 1989). The universally distributed, filamentous, nonheterocystous cyanobacterium *Microcoleus* sp. produces colonies containing many filaments usually embedded in a common sheath (Rippka et al. 1979). These colonies are then attached to a surface and form a biofilm. Black mangrove pneumatophores (aerial roots) in Balandra lagoon, Baja California Sur, Mexico, are also a natural habitat of a N_2-fixing *Microcoleus* sp. (Toledo et al. 1995).

The aims of the present study were to observe the pattern of in vitro root colonization in black mangrove seedlings after inoculation with the N_2-fixing cyanobacterium *Microcoleus* sp. and to measure the N_2 fixation occurring on the roots.

Materials and methods

Plant material and growth conditions

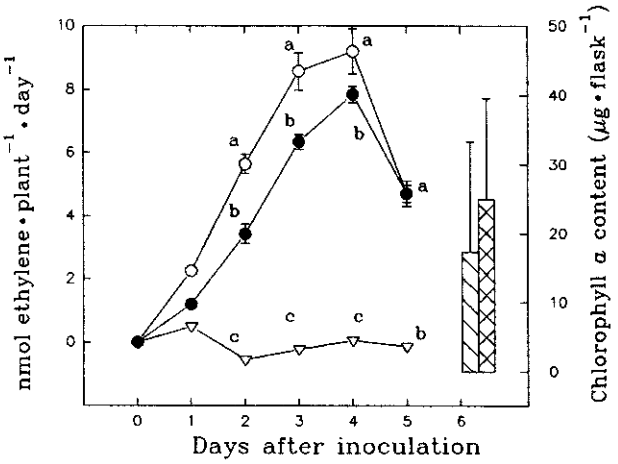
We collected black mangrove propagules (dispersal vegetative units) *(Avicennia germinans* (L.) Stern) from the same locality of the in situ N_2-fixation measurements described in our previous paper (Toledo et al. 1995) and from approximately the same trees. The seasonal production of propagules began at Balandra lagoon in August 1993 and lasted for approximately 40 days. Propagules measuring 4 cm were directly picked from the trees, transferred to the laboratory, and examined; those with small insect holes were discarded. The disinfection process was done by serial washing, first with Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma Chemical Corp., St. Louis, Mo.) (2% v/v) for 5 min, followed by rinsing with sterile tap water. Later, propagules containing the pericarp were treated with 1 % NaOCI (Clorox, Mexico City) for 5 min. Then, the propagules were peeled with a sterile scalpel and the pericarp was discarded. The naked propagules were further disinfected with 0.1 % NaOCl and finally rinsed with sterile tap water six times. All these steps were carried out under sterile conditions. The efficiency of our disinfection process was checked by light and scanning electron microscopy, as well as by plating on common bacterial media. No N_2-fixing microorganisms were found to be associated with the disinfected propagules (Toledo et al. 1995).

White sand was collected from a sandy beach in Balandra lagoon and washed 10-12 times with pressurized salty tap water (2560 $\mu S \cdot cm^{-1}$) until the discarded supernatant was completely clear. The wet sand was then incubated at room temperature (28-33°C) for 24 h to allow time for the spores of any microorganisms to germinate. Afterwards, the sand was spread in a thin layer on a tray and was sterilized in an oven at 250°C overnight. This first sterilization was essential to ensure sand sterilization, which could not be done only with the autoclaving procedure described later. Sand (260 g each) was then losely packed into cylindrical, 900-mL transparent glass beakers (17 x 7.5 cm). Each beaker was supplemented with the mineral salt solution plus vitamins of the Murashige and Skoog (1962) medium containing 25 g sodium chloride $\cdot L^{-1}$ and the following (mg $\cdot L^{-1}$): NH_4NO_3, 1650; KNO_3, 1900; KH_2PO_4, 170; $MgSO_4 \cdot 7H_2O$, 370; $CaCl_2 \cdot 2H_20$, 440; $FeSO_4 \cdot 7H_2O$, 27.8; Na-EDTA, 37.3; H_3BO_3, 1.55; $MnSO_4 \cdot H_2O$, 4.22; $ZnSO_4 \cdot 7H_2O$, 2.15; KI, 2; $NaMoO_4 \cdot 2H_2O$, 0.0735; $CUSO_4 \cdot 5H_2O$, 0.125; $CoCl_2 \cdot 6H_2O$, 0.125; glycine, 0.02; myoinositol, 1; nicotinic acid, 0.005; pyridoxine monochloride, 0.005; thiamine hydrochloride, 0.001. The volume of the solution in the beakers was adjusted to maintain approximately 3 mm of water over the sand surface. The beakers were then covered with thick aluminum foil and autoclaved for 20 min. Afterwards, the foils were further fastened and sealed with elastic bands. All these procedures were essential to obtain sterile assemblies in which to grow the plants. Three disinfected propagules were then planted 1 cm deep in the sand of each beaker with sterile forceps. The plants were grown at 22 ± 1 °C in a growth chamber under 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of fluorescent light for 1 month at 12-h light:dark photoperiods. Fresh mineral medium was added only when required to maintain the level of solution in each beaker. The beakers were tall enough and the whole plants were enclosed in the foil-sealed beakers. All procedures were routinely carried out under aseptic conditions.

Cyanobacterial cultures

Microcoleus sp. B 1 used for inoculation was isolated from a black mangrove pneumatophore and grown in N-free ASN-III medium (Rippka et al. 1979) as described in the previous paper (Toledo et al. 1995). The cyanobacterial culture was characterized as an undefined mass, composed of numerous filaments and resembling a mat. First, it was washed in sterile N-free ASN-III, and then, it was homogenized in a sterile tissue homogenizer (model 5VB, LFE, U.S.A.) to obtain a cell suspension. The suspension was centrifuged at 3000 X g for 10 min to discard disrupted filaments suspended in the supernatant. The pellet was resuspended in the same medium and adjusted to an optical density of 0.27 at 540 nm. This suspension was used as the inoculum for our experiments. This inoculum concentration was equivalent to 1.48 mg chlorophyll *a* $\cdot mL^{-1}$ and about 1.16 x 10^4 filaments $\cdot mL^{-1}$ (counted in a hemocytometer under light microscopy). Each filament consisted of four or five cells.

Fig. 1. Nitrogen fixation of inoculated *Avicennia germinans* seedlings with *Microcoleus* sp. Columns represent the amount of chlorophyll *a* and bars represent the standard deviation. Points (for each day, separately) followed with a different letter differ significantly at $P \leq 0.05$ by one-way ANOVA. Bars at each ethylene determination represent standard errors. ○, inoculated plants; ●, cyanobacteria alone; ▽, noninoculated plants; ▨, inoculated plants; ▧, cyanobacteria alone.



N_2 fixation on inoculated plantlets

One-month-old plants were selected for each experiment. The plants selected were always from the same lot, were about the same height (approximately 100 mm), and had a similar number and size of true leaves. Prior to inoculation, the roots were carefully washed in 40 mL of sterile N-free ASN-III medium in a beaker to remove sand particles. No sand particles remained on the roots. Then, they were transferred to 250-mL filtration flasks equipped with a cotton plug at the top, a rubber septum at the side-arm exit, and 25 mL of N-free ASN-III medium containing cyanobacterial inoculum. The plants were first incubated for 24 h under the described growth conditions to allow attachment to the roots by the cyanobacteria. The noninoculated plants were similarly incubated in 25 mL of sterile N-free ASN-III. Twenty-four hours after inoculation, an additional 25 mL of N-free ASN-III medium containing 1.6% agar (Sigma) at approximately 38°C was added to each flask. The mixture produced a semisolid medium (0.8% agar) in the filtration flask upon partial solidification. Then, the cotton plugs were removed and the flasks were sealed with rubber stoppers. Ten millilitres of air were replaced by pure acetylene. Air samples were drawn with 1-mL sterile syringes immediately after the acetylene injection and every 24 h thereafter for 5 days. The air samples were collected in 5-mL serum bottles, which were sealed with rubber septums. The analysis of the samples was done in a Varian 6000 gas chromatograph (Varian Instrument Group, U.S.A.) as described earlier (Toledo et al. 1995). The amount of ethylene produced was expressed as nanomoles of ethylene per 24 h per plant.

Parallel to the N_2-fixation determinations, additional replicates (but without acetylene) were selected to examine cyanobacterial root colonization using phase contrast microscopy and scanning electron microscopic (SEM) analyses. The roots were cut with a razor blade and small root pieces (10 mm each) were selected with the aid of a dissecting microscope. These root pieces were first shaken in a vortex mixer for 1 min at maximum speed to remove nonattached filaments and cells, and then rinsed with sterile liquid ASN-III medium. Preparation of the samples for SEM was described earlier (Toledo et al. 1995).

At the end of the experiment, chlorophyll *a* was extracted from the semisolid remaining medium and the roots, by cutting each root into three pieces. Acetone (450 mL, histological grade; Fisher Scientific Co., Fair Lawn, N.J.) was added to 50 mL of suspension (roots and medium). The extract was incubated 17 h at 4°C in the dark and centrifugated at 2800 x g for 10 min. The supernatant was measured spectrophotometrically at 665 nm. The amount of chlorophyll *a* was calculated according to the equation of Tandeu de Marsac and Houmard (1988).

Experimental design and statistical analysis

The N_2-fixation experiment was repeated five times, each with eight replicates. A replicate consisted of one filtration flask containing one plant. Controls consisted of uninoculated plants, the same concentration of inoculated cyanobacteria in ASN-III medium without plants, and flasks containing the different gases alone. The results are expressed as the mean values of all experiments. One-way analysis of variance (ANOVA) was performed at $P \leq 0.05$.

SEM observations were carried out on five replicates per sampling date. Each replicate consisted of five root pieces. The total number of samples observed was 150.

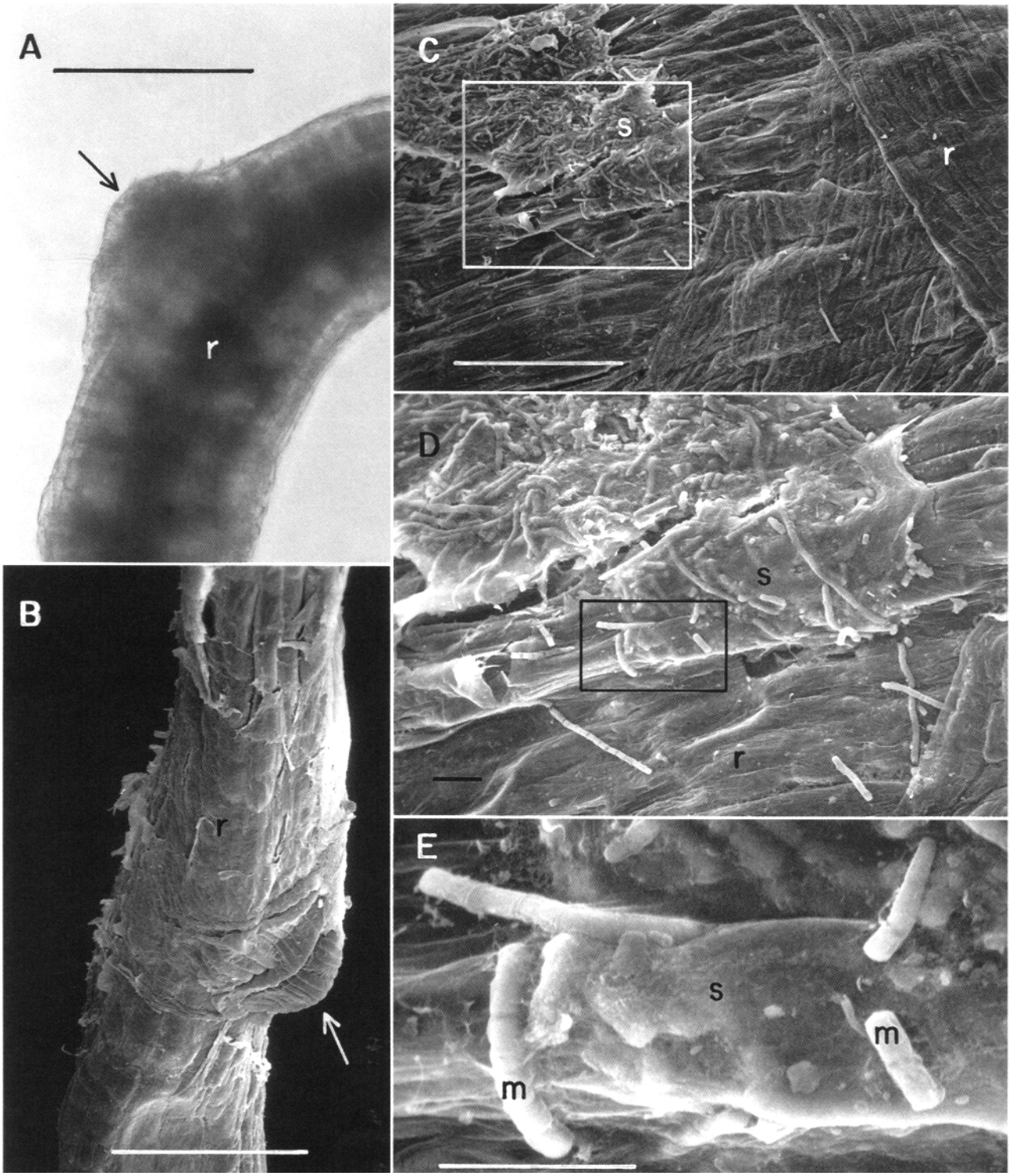
Results and discussion

Any evaluation of interactions between plants and microorganism should test the following questions. (*i*) Is the microorganism capable of colonizing the host plant? This is a basic requirement established for many terrestrial plant-bacteria interactions (Parke 1991). (*ii*) Does the microbe excrete any potentially beneficial substance for plants or help the plant scavenge needed minerals during the association (Alexander and Zuberer 1993; Bashan et al. 1990; Li et al. 1992; Mazzola et al. 1992; Neilands and Leong 1986; Paula et al. 1992)? (*iii*) Does the microbe survive long enough and in sufficient numbers to become a possible agent for further inoculation evaluations (Bashan 1993; Bashan and Levanony 1990; Lynch 1990)?

Mangrove plants are always associated with cyanobacteria of diverse species. The nature of the association (whether beneficial or not) is unknown, although it is visibly clear that the interaction is at least nonpathogenic. It is also unknown whether cyanobacteria participate in the life cycle of mangrove plants, although dense colonization of the submerged parts of mangrove trees is frequently observed (Dor and Levy 1984; Potts 1979; Sheridan 1991; Toledo et al. 1995).

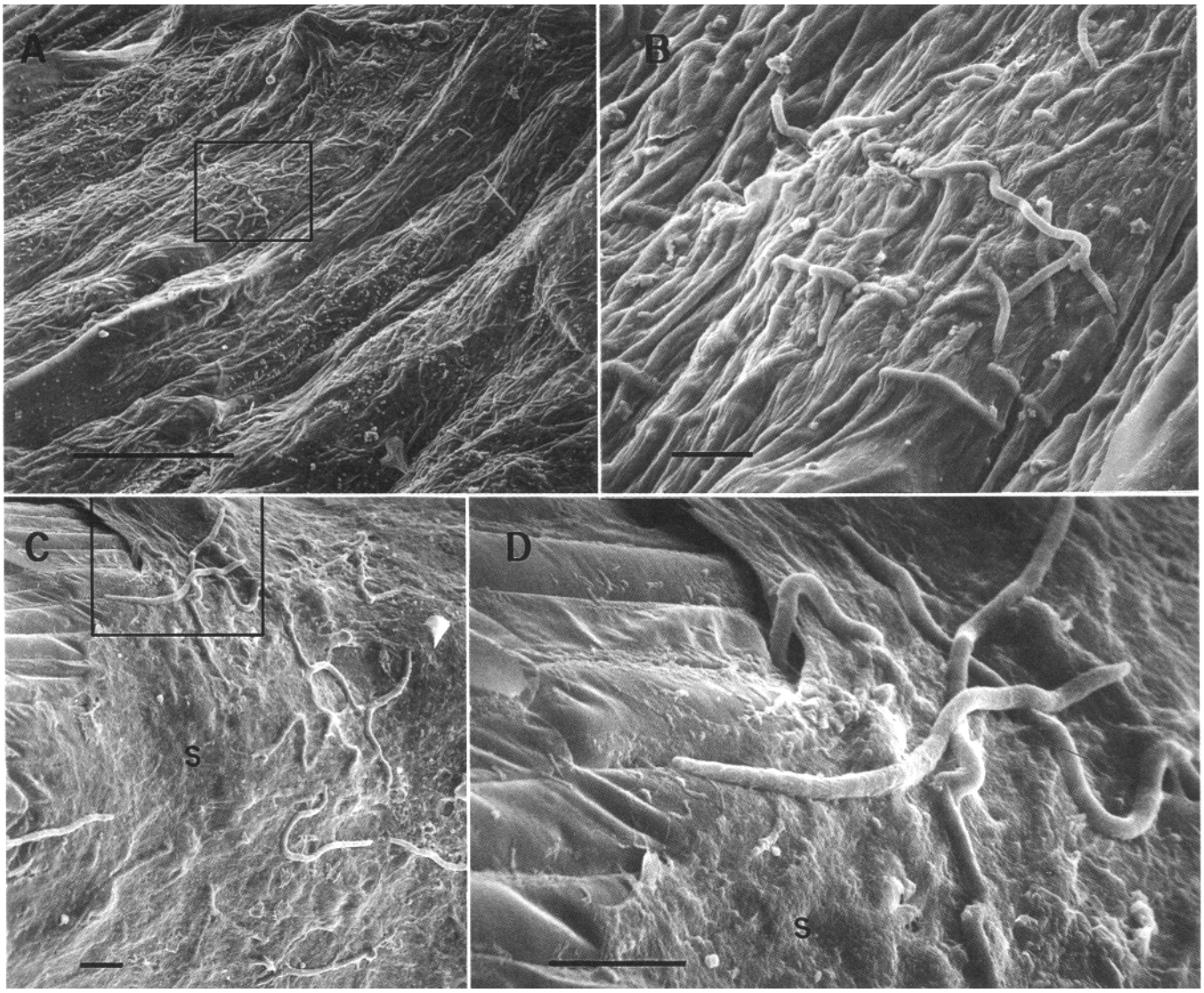
In this study we used an isolate of cyanobacteria originally from an aerial root (pneumatophore) to inoculate roots. The logic behind it was based on the following. (*i*) *Microcoleus* sp. is the most abundant cyanobacteria in the Balandra mangrove ecosystem. As such, the probability is the greatest that, when a propagule falls from the tree to the nearby sediment or lagoon water, it will be colonized by this cyanobacterial genus.

Fig. 2. (A) The colonization process along the root surface by *Microcoleus sp. I* day after inoculation: light microscopy of pocket-like colony (arrow). (B) SEM of an apparent similar colony (arrow). (C-E) Detailed formation of cyanobacterial filaments embedded in a common biofilm on the root surface. (D and E) Inserts show higher magnification of the original colony (in C). Scale bars = 10 µm (A, B. D. E) and 100 µm (C). in, *Microcoleus* sp. filaments: *r.* Uncolonized root: *s*, biofilm.



(*ii*) *Microcoleus* is associated with any submerged mangrove plant part, and (*iii*) it is unknown whether artificial inoculation of cyanobacteria should be on the aerial or subterranean part of the seedling to get maximum benefit for the plant. It is definitely simpler to inoculate roots and to maintain a live population of cyanobacteria there.

Fig. 3. (A) On the 2nd day of colonization, small colonies merged into larger colonies. (B) Insert in A showing numerous filaments on the root surface. (C) Three days after inoculation *Microcoleus* sp. filaments are embedded in a large biofilm. (D) Insert in C showing filaments moving in and out of the biofilm. Scale bar = 100 µm (A) and 10 µm (B-D). *s*, biofilm.

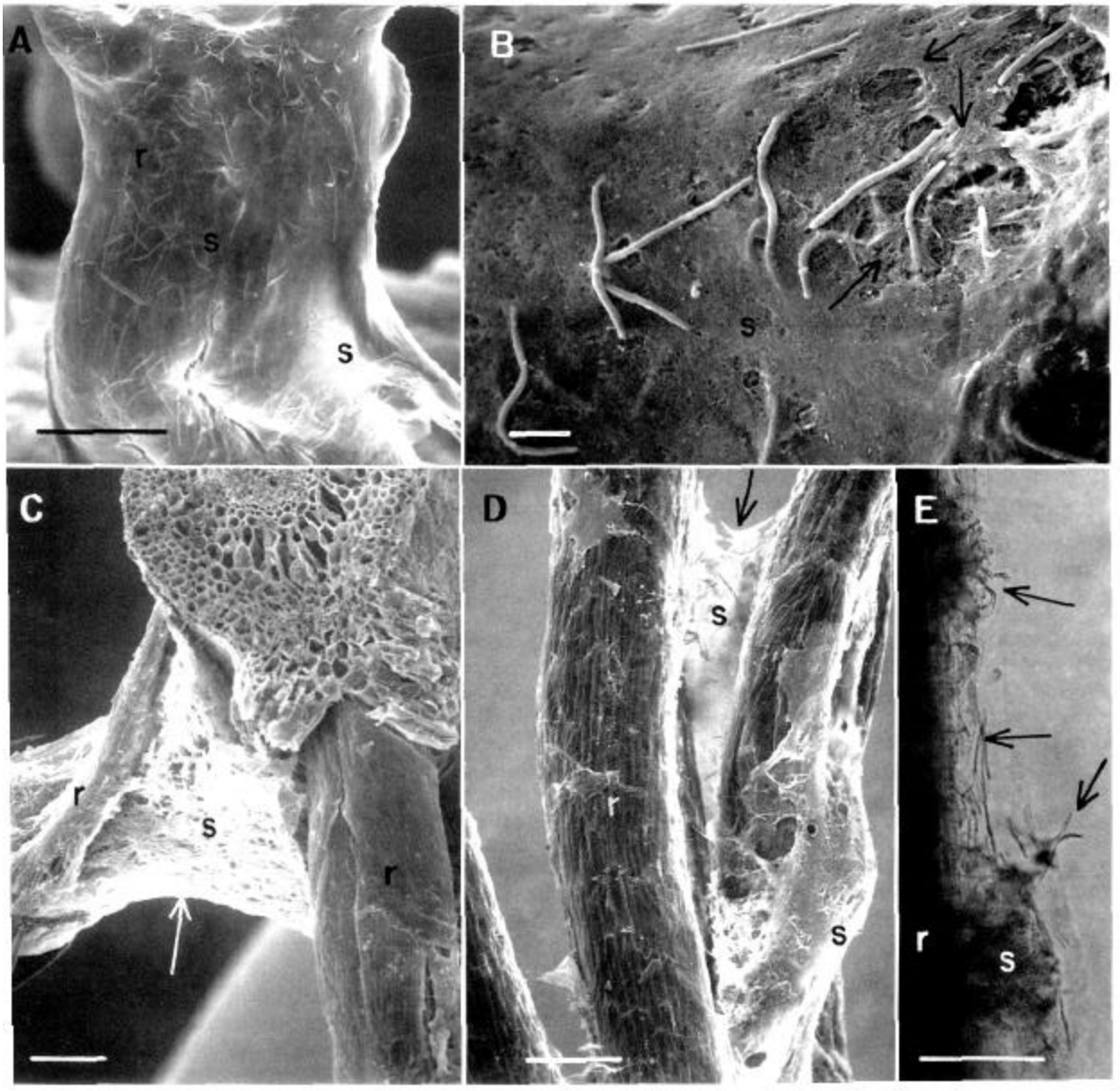


The goal of the present study was to explore the possibility that black mangrove plants may associate with cyanobacteria from the onset of propagule establishment and to present, to the best of our knowledge, the first report on inoculation of marine plants with potentially beneficial cyanobacteria.

In closed-system experiments, N_2 fixation gradually increased when the plants were inoculated with the cyanobacteria. However, 5 days after inoculation there was a sharp, statistically significant, decrease in this activity for all repetitions (Fig. 1). This decrease could be the result of either a loss of nutrients (such as phosphorous) for the plant or the buildup of yet undefined inhibitors.

Although the cyanobacterial growth appeared denser in the beakers containing plants compared with cyanobacteria in nutrient solution alone, the chlorophyll *a* determinations to evaluate the quantity of cyanobacteria on the root varied greatly (Fig. 1, columns). A possible explanation for the enhanced growth of the cyanobacteria might be that there is some heterotrophic metabolism involved, in which carbon from the roots is somehow used to support nitrogenase activity. It should be noted that filamentous cyanobacteria are almost always associated with other bacteria (Marshall 1989). The isolation method we used (Stal and Krumbein 1985) cannot exclude the possibility that *Microcoleus* sp. biofilms also contained endogenous undetected N_2-fixing bacteria. However, (*i*) in numerous SEM micrographs of inoculated roots taken in this study, hardly any bacteria other than cyanobacteria were detected, (*ii*) no bacteria other than cyanobacteria were detected during inoculum preparation, (*iii*) the mangrove propagules underwent a rigorous surface-dis infection process and no endophytic N_2-fixing bacteria were ever isolated from these types of propagules, and (*iv*) inoculation of cyanobacteria into nutrient broth or N-free media did not yield other bacterial populations.

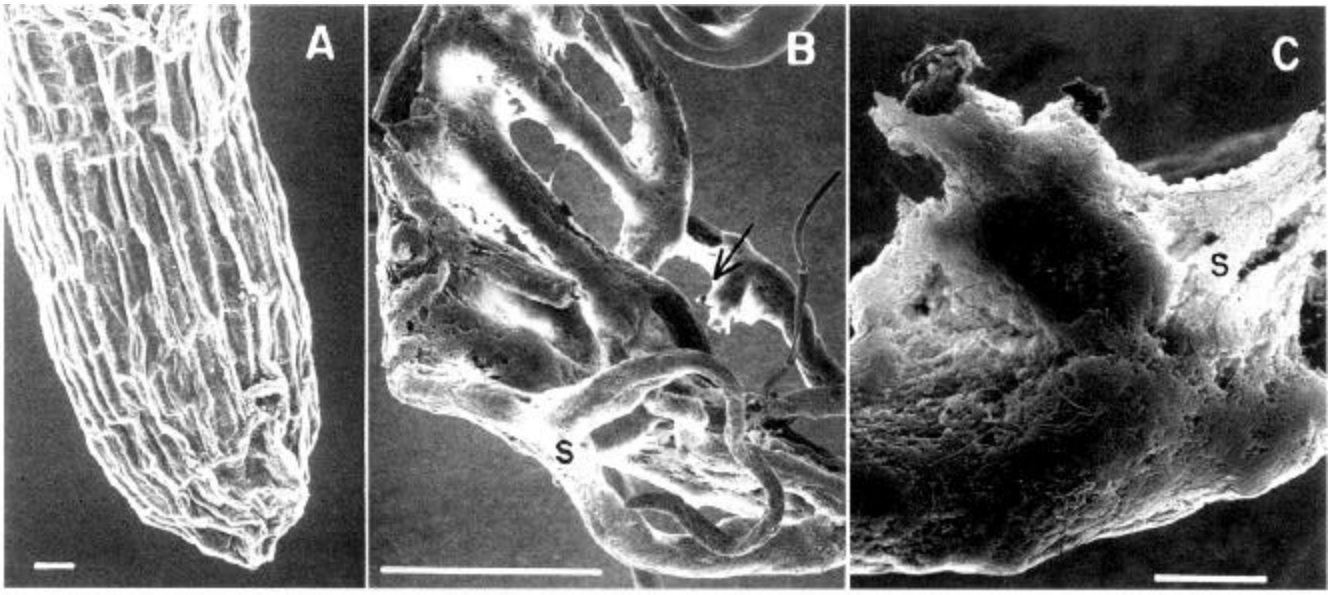
Fig. 4. (A) SEM 4 days after inoculation, cyanobacteria embedded in thick biofilm covered the entire small root. (B) A multilayer of biofilm (arrows) embedded the cyanobacterial filaments. (C) A heavy biofilm production in site of emergence of new roots (arrow), and (D) gluing two small roots (arrow) 5 days after inoculation. (E) Light microscopy of biofilm pocket-like colony (like in D) showing embedding and releasing of cyanobacteria from the biofilm (arrows). Scale bars = 100 µm (A, D) and 10 µm (B, C, *E). r,* root; s, biofilm containing *Microcoleus* sp. filaments.



The main feature of the colonization under a noncompetitive environment was the production of biofilm. The cyanobacterial colonization process can start on any part of the root but was mainly observed in the root elongation zone, in places where lateral roots emerge, or on the root cap (light microscopy, data is not shown). In the first 24 h, the cyanobacterial filaments established small, pocket-like colonies (Figs. 2A and 213) on many secondary roots. The fine structure of colonization was a biofilm (or gelatinous matrix) in which many filaments were embedded (Figs. 2C-2E).

On the 2nd day after inoculation, the colonies became denser and cyanobacterial aggregates covered a large portion of the root surface (Figs. 3A and 3B). On the 3rd day, about 50-60% of the root surface area was covered with this biofilm (light microscopy, data not shown). The biofilm formed a thick layer, wrapping the entire root by submerging almost all the

Fig. 5. SEM of root colonization by cyanobacteria 6 days after inoculation. (A) Noninoculated root tip. (B) Representative part of the root system of a seedling completely covered with biofilm. (C) Enlargement of the arrow site in B showing massive multilayer biofilm developed on the root. Scale bars = 10 µm (A), 1 mm (B), and 100 µm (C). *s*, biofilm.



former separate small colonies (Figs. 3C and 3D). SEM observations, and especially light microscopy, of live specimen revealed the movement of filaments in and out of the biofilm (Figs. 3C, 3D, and 4E). It can be proposed that this may allow the cyanobacteria to establish the small new colonies we observed nearby on the roots (light microscopy, data not shown). Four days after inoculation, the filaments produced extensive multilayered biofilm that completely covered small roots (Figs. 4A and 4B). On the 5th day, colonization was apparent to the naked eye. The macroaggregates were located mainly in the places where new roots emerged (Fig. 4C, arrow) or they glued together several small secondary roots (Fig. 4D, arrow). This gluing effect might be caused by a mixture of biofilm material from cyanobacterial origin and mucilage from plant origin, but probably not from the semisolid agar in which the plants were embedded during the colonization process since it hardly formed a gel. Six days after inoculation, the entire root system was embedded in a thick biofilm (Figs. 5B and 5C) in contrast to noncolonized roots (Fig. 5A). No negative effects on plant growth were visibly observed as a result of this massive colonization. On the contrary, the inoculated plantlets were greener and slightly larger than noninoculated plants (data not shown).

Although this is an in vitro study, the extensive biofilm formation observed along the roots may lead to the following speculations, which need to be further evaluated under field conditions. (*i*) The biofilm may have allowed cyanobacteria to build large macrocolonies, thereby maintaining a suitable microenvironment for growth, similar to cyanobacteria on aquatic duckweed plants (Zuberer 1984). (*ii*) Biofilms are also known to absorb water (Dor and Levy 1984). The submerged parts of black mangrove and surface of surrounding sediments are subjected to daily desiccation (Jones 1992; Potts 1979, 1980; Potts and Whitton 1980), known to inhibit N_2 fixation by cyanobacteria (Pons 1980). Therefore, by producing biofilms, the cyanobacteria increase the duration of the wet conditions, which may provide prolonged periods for N_2 fixation. (*iii*) At the same time, a thick biofilm may slow down the release of cyanobacterial-produced compounds into the lagoon water and the open sea.

Cyanobacteria, by virtue of their abundance in the mangrove forests and their high capacity of N_2 fixation (Pons 1979,1984), are natural candidates for the evaluation of seedling inoculation for future reforestation and the rehabilitiation of destroyed coastal lagoons (G. Toledo and Y Bashan, unpublis hed data). At this time, it is unclear whether the nitrogen is directly transferred from the cyanobacteria to the mangrove seedling since ^{15}N assimilation tests have not yet been done.

On December 1994, 500 nursery-reared black mangrove seedlings were transplanted into a disturbed area in Balandra lagoon. Seven months later, 76.5% of them survived in nature without any apparent negative effects of the transplantation (G. Toledo and Y. Bashan, unpublished data). Thus, our inoculation study points out the possibility of using cyanobacteria as a future inoculant for mangrove nurseries.

In summary, this study shows that the cyanobacteria *Microcoleus* sp. under a noncompetitive environment in closed system experiments intensively colonizes the roots of black mangrove plants. During the association, N_2 fixation on the plant roots increased.

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