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In vitro transfer of fixed nitrogen from diazotrophic filamentous cyanobacteria to black mangrove seedlings

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Abstract

Two isolates of the diazotrophic filamentous cyanobacterium *Microcoleus chthonoplastes*, one obtained from black mangrove (*Avicennia germinans*) pneumatophores and one from cyanobacterial mats, were inoculated onto young mangrove seedlings to evaluate nitrogen transfer from the bacterium to the plants under in vitro conditions in closed system experiments. Total nitrogen and ^{15}N incorporation in plant parts were measured. The levels of total N and ^{15}N in the inoculated leaves were significantly higher than in noninoculated plants.

Keywords: *Avicennia germinans*; Beneficial bacteria; Black mangrove; Diazotrophic cyanobacteria; *Microcoleus chthonoplastes*; Nitrogen fixation; Plant growth promoting bacteria

1. Introduction

Mangrove forests are indispensable for sustainable coastal fisheries in the tropics. They are a major refuge and feeding ground for numerous economically important fish and shellfish [1]. Once a mangrove forest has been cleared, the coastal fishing may be irreversible damaged [2], therefore, attempts have been made to reforest mangroves [3,4]. The natural reforestation rate of black mangroves (*Avicennia germinans* (L.) Stern) in arid zones is slow [5]. Artificial reforestation, with or without cyanobacteria inoculation, might be a solution [4]. Studies of cyanobacteria inoculation of plants have been concentrated on rice [6–10], wheat [11–13] and black mangroves [14].

Marine cyanobacterial populations are an integral and a major component of the microbiota in every mangrove ecosystem [15–18]. They colonize any submerged surface: sediment, roots, aerial roots, branches, and trunks of mangrove [19–21]. The universally distributed, filamentous, nonheterocystous cyanobacteria *Microcoleus* spp. produce colonies containing many filaments, usually embedded in a common sheath [22]. They are also the most abundant cyanobacteria in the Balandra mangrove eco-

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system in Baja California Sur, Mexico [18]. Although *Microcoleus* sp. isolated from microbial mats failed to fix any nitrogen because they lack at least one of the requisite genes for this process [23], our mangrove strain did [14]. The exact interaction between cyanobacteria and mangroves or any resulting mutual benefits have yet to be established.

A general diazotrophic activity in mangroves has been observed [21,24–26]. A previous study showed *Microcoleus chthonoplastes* inoculation increased N_2 fixation activities on black mangrove roots [14]. The aim of the present study was to measure the possible transfer of fixed nitrogen to the plants after inoculation using two strains of diazotrophic *M. chthonoplastes*.

2. Materials and methods

2.1. Plant material and growth conditions

We collected black mangrove propagules (disperse vegetative units) for three consecutive years from Laguna de Balandra [24] and from two locations in western Ensenada de La Paz (1 km south and 4 km north of The Center for Biological Research of the Northwest (CIB)). The seasonal production of propagules in these sites began in August, and lasted for approximately 40 days. The preparation of the propagules for inoculation was as described by Toledo et al. [14].

Fine white sand was collected from a sandy beach in Ensenada de La Paz and was washed 10–12 times with pressurized salty tap water (2560 μmhos cm^{-1}) until the discarded supernatant was completely clear. The wet sand was incubated at room temperature (28–33°C) for 24 h in a 1 N HCl bath to eliminate organic matter and germinating spores. The acid washed sand was then rinsed with tap water until the supernatant was pH 7.0 and given a final wash with reverse osmosis drinking water (325 μmhos cm^{-1}; equiv. 0.2 g salts l^{-1}). Afterwards, the sand was spread in a thin layer on a tray and heated in an oven at 250°C for 2 h with constant air circulation. This presterilization procedure was essential to ensure sand sterilization which could not be assured by use of an autoclave alone. Presterilized sand (260 g, 5 cm depth) was then loosely packed into cylindrical, 900-ml transparent glass beakers (17×7.5 cm). Each beaker was saturated with the mineral salt plus vitamins medium (MS-NaCl [27]) that contains the following: (in mg 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; $FeSO_4 \cdot 7H_20$, 27.8; Na-EDTA, 37.3; H_3BO_3, 1.55; $MnSO_4 \cdot H_2O$, 4.22; $ZnSO_4 \cdot 7H_20$, 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;, *myo*-inositol, 1; nicotinic acid, 0.005; pyridoxine monochloride, 0.005; thiamine hydrochloride, 0.001; and 25 g l^{-1} of NaCl. The volume of the solution in the beakers was adjusted to maintain approximately 3 mm of solution over the sand surface. The beakers were then covered with thick aluminum foil and autoclaved for 20 min. Afterwards, the foil was further fastened and sealed with elastic bands. All these procedures were essential to obtain sterile assemblies in which to grow the plants. About 300 disinfected propagules were preincubated in 100-ml flasks containing 50 ml MS-NaCl medium at 24–26°C and constant light of 100 μmol m^{-2} s^{-1} to check for contamination. The propagules were observed daily and flasks containing contaminated propagules were discarded. After 8 days, the propagules were removed and three noncontaminated propagules were planted 1 cm deep in the sand of each beaker by using sterile forceps. The plantlets were grown at 26 ± 1°C in a growth chamber (Biotranette mark III Environmental Chamber, Lab Line Instruments, Melrose Park, IL) under 50 μmol m^{-2} s^{-1} of fluorescent light for 1 month with a 12-h (light:dark) photoperiod. Fresh mineral medium was added only when required to maintain the level of solution in each beaker. The beakers were tall enough so that the whole plants were enclosed in the foil sealed beakers. All procedures were routinely done under aseptic conditions.

2.2. Cyanobacterial cultures

Cyanobacterial strains from two sources were compared. *M. chthonoplastes* strains B1, isolated from the black mangrove pneumatophores (aerial roots), and strain SC7B9002-1, isolated from cyanobacterial mats [28] were grown in ASN-III medium [29] for 10 days as described before [14]. Both strains were diazotrophic. Since we used axenic cultures,

they had no accompanying contaminating diazotrophs. Under identical experimental conditions described in this study, strain B1 reduced acetylene producing 6.1 nmol ethylene per culture per day (after 3 days of culturing). When inoculated onto black mangrove roots, the rate of acetylene reduction increased to 9.2 nmol ethylene per plant per day. Strain B1 was also able to heavily colonize the seedling roots [14]. 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, then homogenized in a sterile tissue homogenizer (Caframo R2R1, Wiarton, Ont., Canada) to obtain a cell suspension. The suspension was centrifuged at $3000 \times g$ 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 ml^{-1} of chlorophyll *a* and to about 1.16×10^4 filaments per ml (counted in a hemocytometer under light microscopy). Each filament consisted of four to five cells.

2.3. Inoculation of plantlets

One-month-old sand culture plantlets were selected for each experiment. The plants selected were always from the same lot, 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 to remove all sand particles. Washed plantlets 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 sterile N-free ASN-III medium containing cyanobacterial inoculum was added. The plants were first incubated for 24 h under the described growth conditions to allow attachment to the roots by the cyanobacteria. Plants suffering from the transfer or contaminated flasks were discarded. The noninoculated plants were similarly incubated in 25 ml of sterile ASN-III but containing an additional 0.75 g l^{-1} $NaNO_3$ (to avoid stress of nitrogen starvation of noninoculated plantlets). Twenty-four hours after inoculation, an additional 25 ml of N-free ASN-III medium was added to each flask, the cotton plugs were removed, and the flasks were sealed with rubber stoppers, which were secured by several layers of parafilm to prevent gas leakage. 15 ml of air was replaced with ^{15}N gas (Aldrich, Milwaukee, WI). The flasks were gently mixed for 1 h at 120 rpm on a rotary shaker to allow a better dissolution of the added gas into the liquid medium containing the cyanobacteria inoculum. The flasks were incubated for 14 days without movement in a growth chamber under the conditions described earlier. Every 3 days, the flasks were opened, ventilated, and a new dose of 15 ml ^{15}N gas was added. For analysis of the atmosphere in the incubation flasks, 1-ml samples were removed and put into a 5-ml serum vial as follows: from the sealed serum vial, 1 ml of air was removed. To the sealed vial, 1 ml of the atmosphere from the incubation flasks was then added. As a precaution, samples were taken and analyzed from the commercial ^{15}N tank (to ensure the level of ^{15}N gas), before every ventilation of the flasks (as an assurance the flask was not leaking), and after every injection of a new dose of ^{15}N gas into the incubating flasks, from three flasks chosen at random.

Table 1
Total nitrogen concentration (in percentage) in leaves and stems of black mangrove seedlings after inoculation with *M. chthonoplastes* B1[a]

Plant part	Noninoculated		Inoculated			
	Without $NaNO_3$[b]	With $NaNO_3$	Without $NaNO_3$	With $NaNO_3$	% of noninoculated without $NaNO_3$	% of noninoculated with $NaNO_3$
Leaves	1.40b[c]	2.99a	3.00a	3.84a	214.00	128.00
Stems	1.74a	1.99b	1.90b	2.00b	109.00	101.00

[a]Average of two independent experiments done in 1994 and in 1995 using propagules from Laguna de Balandra.
[b]0.75% (w/v).
[c]Numbers in the same row denoted by a different letter differ significantly at $P \leq 0.05$ in Student's *t*-test.

Table 2
Total nitrogen concentration (in percentage) in roots, leaves and stems of black mangrove seedlings after inoculation with *M. chthonoplastes* strains B1 and SC7B9002-1[a]

Plant part	Cyanobacteria strain		B1	SC7B9002-1	
	noninoculated	inoculated	% of noninoculated	noninoculated	% of noninoculated
Roots	1.3a[b]	1.43a	110.00	1.36a	105.00
Stems	2.49a	2.44a	98.00	2.58a	103.00
Leaves	3.97a	4.50b	113.00	4.17 ab	105.00

[a]Experiment done in 1996 using propagules from Ensenada de La Paz.
[b]Numbers in each plant part in the same row denoted by a different letter differ significantly at $P \leq 0.05$ in single factor ANOVA.

2.4. Nitrogen determinations

At the end of each experiment, the plants were removed and each plant was separated into leaves, stem, and roots. Each plant part was dried (separately) in an oven at 60°C for 12 h, milled to a fine powder, and packed into Eppendorf tubes. The total nitrogen content was measured by automatic micro-Kjeldahl after digestion (Digestion System 12.1009, and Kjeltec Auto 1030 Analyzer, Tecator, Höganäs, Sweden). Abundance of ^{15}N in the samples was measured by automated isotope ratio mass spectrometry (Tracermass, Europa Scientific, Crewe, UK) according to standard methods [30] and expressed as ^{15}N in parts per thousand (δ).

2.5. Experimental design and statistical analysis

The experiment was repeated three times in successive years, each with 10 replicates. Total N was determined in 1994, 1995, and 1996 experiments though ^{15}N was determined only in 1996. A replicate consisted of one filtration flask containing one plant. Controls consisted of noninoculated plants, and the same concentration of inoculated cyanobacteria in ASN-III medium without plants and flasks containing ^{15}N gas. The results were analyzed by either single factor analysis of variance (ANOVA) or Student's *t*-test at $P \leq 0.05$.

3. Results and discussion

Mangrove plants are always associated with cyanobacteria of diverse species [16,18,20,31]. The nature of the association (whether beneficial or not) is unknown, although it is visibly clear the interaction is nonpathogenic. It is also unknown whether cyanobacteria participate in the life cycle of mangrove plants. A previous study showed that N_2 fixation increased in the roots of black mangrove seedlings after inoculation with an isolate of *Microcoleus* [14]. The goal of the present study was to explore the possibility that black mangrove plants are gaining nitrogen as a result of their association with *M. chthonoplastes*.

By repeating a similar experiment three times, we were able to demonstrate that inoculation with cyanobacteria significantly increased the total N content of the leaves of inoculated plants over noninoculated ones (Tables 1 and 2) in the range of 5–114%. This increase in total N was corroborated by ^{15}N incorporation studies (Table 3). The levels of ^{15}N labeling were low because the concentration of ^{15}N in the commercial tanks used was lower than expected, which resulted in the headspace being labeled at only 0.3924 ± 0.129 atom % ^{15}N ($\delta^{15}N = 71‰$; $n = 24$). The total N content increase was insignificant in stems and roots and was independent of the origin of

Table 3
$\delta^{15}N$ in roots, leaves and stems of black mangrove seedlings after inoculation with *M. chthonoplastes* strains B1 and SC7B9002-1[a]

Plant part	Cyanobacteria strain noninoculated	B1 inoculated	SC7B9002-1 inoculated
Roots	3.32a[b]	12.26a	7.03a
Stems	2.37a	2.18a	2.04a
Leaves	5.89a	9.98b	10.92b

[a]Experiment done in 1996 using propagules from Ensenada de La Paz.
[b]Numbers in each plant part in the same row denoted by a different letter differ significantly at $P \leq 0.05$ in single factor ANOVA. $\delta^{15}N$ of $NaNO_3$ supplement to noninoculated plants was not measured and was assumed to be near natural abundance (1–5‰).

the propagules (compare Tables 1 and 2). Although the difference between inoculated and noninoculated plants is larger when the plants are incubated in an N-free medium, we preferred to supplement the medium of noninoculated plants with a low level of nitrogen to prevent undesirable effects of nitrogen starvation, which visibly affect the plants. The need to supplement a low level of nitrogen to noninoculated plants to obtain meaningful comparison between inoculated and noninoculated plants is well documented for inoculation of terrestrial plant growth promoting bacteria *Azospirillum* sp. on many crop plants [32].

Cyanobacteria, by virtue of their abundance in the mangrove forests and their high capacity for N_2 fixation [25], are natural candidates for the evaluation of seedling inoculation for future reforestation and rehabilitation of destroyed coastal lagoons [4].

In summary, this study shows that inoculation with the cyanobacteria *M. chthonoplastes*, under a noncompetitive environment in closed-system experiments increased the nitrogen content of black mangrove seedlings, probably by N_2 fixation.

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