MUTUALISM BETWEEN *PHYLLOBACTERIUM* SP. (N_2-FIXER) AND *BACILLUS LICHENIFORMIS* (P-SOLUBILIZER) FROM SEMIARID MANGROVE RHIZOSPHERE

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Sediment and rhizosphere microorganisms are the major biological components assuring mangrove productivity (14). Probably because of diverse microbial activity, mangrove ecosystems are one of the three most productive ecosystems together with rain forests and coral reefs. Yet, mangroves have been alarmingly and systematically deforested, similar to rain forests (16, 20). To aid reforestation, inoculation of the seedlings with plant growth-promoting bacteria (PGPB)(3) is suggested (6, 18, 19), similar to what has been done in agriculture (3,10,11,12) and temperate forests (7).

Very little is known about the plant growth promoting bacteria (PGPBs) that have the potential to colonize mangroves and facilitate their growth. The diazotrophic cyanobacterium *Microcoleus chthonoplastes* improved N_2-fixation (19) and nitrogen incorporation in black mangrove seedlings (6). The terrestrial halotolerant *Azospirillum halopraeferens* and halotolerant *A. brasilense* Cd can successfully colonize black mangroves roots in seawater (18). Recently, several PGPBs of mangrove origin promoted the growth of the annual, potential oilseed seaweed *Salicornia bigelovii*, which shares the semiarid mangrove ecosystem with trees (5).

In agricultural and forestry inoculation practices, the mixing of two or more microbial species usually yields better effects on plant growth than the use of a single microorganism (1,8,9). However, this is complicated when mangrove growth is being facilitated since the interactions of potential mangrove PGPBs among themselves, as well as their effect on plant growth, are unknown.

The aim of the present study was to explore the possible in vitro interactions of two potential mangrove PGPBs, the N_2-fixing *Phyllobacterium* sp. and the phosphate-solubilizing bacterium *Bacillus licheniformis.*

Material and Methods.

Phyllobacterium sp. was isolated from the rhizosphere of semiarid black, white, and red mangrove seedlings, and its nitrogen-fixing capacity was assessed as described by Holguin et al. (15). The bacteria was identified using both fatty acid methyl ester (FAME) and 16S rRNA analysis by a commercial service (Acculab, Newark, DE). *Bacillus licheniformis*, originally isolated from black and white mangroves with a phosphate solubilization capacity of 325 mgP/L, was obtained from the CIB culture collection. It was purified and characterized in a previous study (21).

Black mangroves propagules were collected from Laguna de Balandra, Mexico. They were treated and inoculated with the above mentioned bacteria as previously described for other bacterial species (6,19).

All experiments were done in vitro. Bacteria were grown in unbaffled Erlenmeyer flasks on an environmentally-controlled rotary shaker (*B. licheniformis*) at 150 rpm, and without agitation (*Phyllobacterium* sp.) at 30 ± 1^{o}C. Three different types of media were used; (i) the

minimal medium SRSM2 supplemented with 1.1 g $K_2HPO_4 \cdot 3H_2O$ L^{-1} (Sigma) both liquid and solid (21), (ii) N-free minimal medium for marine bacteria (Holguin, G. 1997; Technical report for Consejo Nacional de Ciencia y Tecnologia, Mexico), and (iii) filtered seawater (18).

Nitrogen fixation was measured by the acetylene-reduction assay using gas chromatography after 48 h of mixed incubation (15). Total nitrogen was measured by an automatic micro-Kjeldahl procedure after digestion (Digestion System 12.1009, and Kjeltec Auto 103 analyzer, Tecator, Höganäs, Sweden). The abundance of ^{15}N in the sample was measured by isochrom continuous flow stable isotope mass spectrometer (Micromass, Manchester, UK) according to standard methods (2) and expressed as ^{15}N in parts per thousand (δ) as previously described (6). Phosphate solubilization was done using a modification of the method of Vazquez et al. (21). Light microscopy of freshly mounted cultures and spore staining was done using a Zeiss (Germany) light microscope (x1000).

Results

Light microscopy showed when the two bacterial species were grown on solid medium they formed one morphotype colony containing both species, whereas on liquid medium they grew separately.

Acetylene reduction of mixed cultures revealed that *Phyllobacterium* sp. fixes twice the amount of nitrogen than when it is in pure culture (Fig 1A).

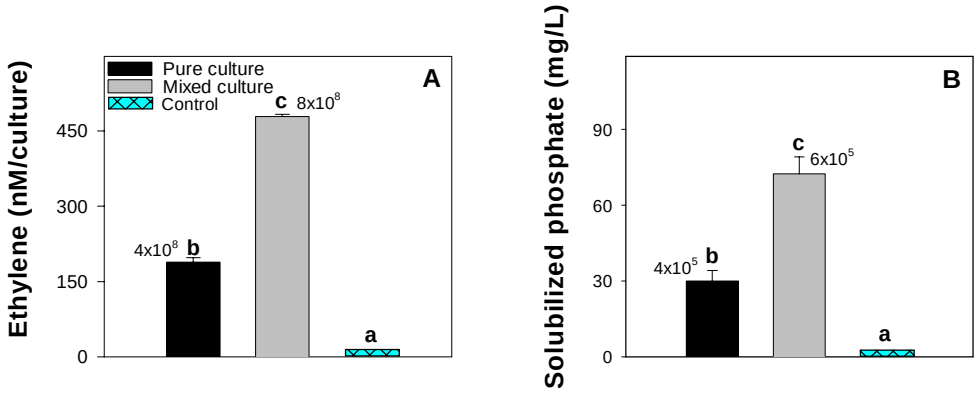

Fig 1. (A) Nitrogen fixation (acetylene reduction) of black mangrove seedlings inoculated with the N_2-fixing bacteria *Phyllobacterium* sp. alone and in mixture with the phosphate solubilizing bacteria *Bacillus licheniformis*. (B) Phosphate solubilization of *B. licheniformis* alone and in mixture with *Phyllobacterium* sp. Columns denoted by a different letter, in each subfigure, differ significantly at $P \leq 0.05$ by the Student's *t*-test. Bars represent Standard Error.

Similarly, the phosphate solubilization activity of *B. licheniformis* in mixed cultures increased significantly over pure culture (Fig 1 B). Black mangrove seedlings inoculated with both pure cultures or mixed cultures showed similar levels of N_2-fixation of 10.7 nmole/culture. All inoculation treatments reduced the nitrogen content in the leaves, stems, and roots of the plants

(Fig 2 A,B,C). However ^{15}N in the leaves and stems of the plants was significantly higher in mixed culture than in uninoculated plants (Fig 2 D,E,F).

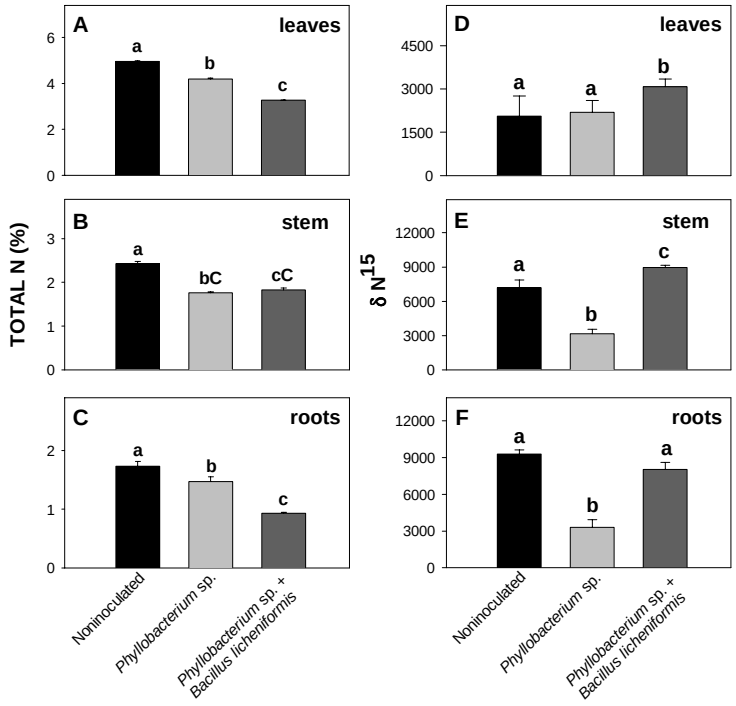

Fig 2. Total Nitrogen content of black mangrove seedlings inoculated with the N_2-fixing bacteria *Phyllobacterium* sp. alone and in mixture with the phosphate solubilizing bacteria *Bacillus licheniformis* (a,b,c). ^{15}N accumulation in the same plants (d,e,f). Columns denoted by a different letter, in each subfigure, differ significantly at $P \leq 0.05$ by by one-way ANOVA. Bars represent Standard Error. Absence of a bar above a column indicates minimal SE.

All inoculations increased the number of leaves of black mangrove seedlings significantly over the uninoculated control although there was no significant difference among the treatments (Fig 3). None of the inoculation treatments affected the root surface area of the seedlings. Root colonization of the seedlings was at the level of $2x10^4$ cfu/g root for both pure cultures and at a level of $1x10^5$ cfu/g root for the mixed culture.

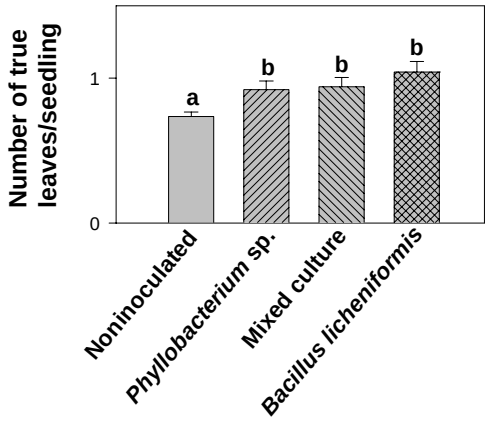

Fig 3. Effect of inoculation with the N_2-fixing bacteria *Phyllobacterium* sp. and the phosphate solubilizing bacteria *Bacillus licheniformis* alone or in mixture on the number of true leaves developed by the seedlings. Columns denoted by a different letter, in each subfigure, differ significantly at $P \leq 0.05$ by by one-way ANOVA. Bars represent Standard Error.

Discussion

Semiarid mangrove forests thrive in lagoons lacking dissolved phosphorus and nitrogen sources (14), essential growth elements for any plant species. Nitrogen fixation is a well documented phenomenon in any mangrove ecosystem (13,17). Many species of microorganisms can solubilize phosphate under marine environments and in white and black mangroves (21). The interactions, if any, between mangrove N_2-fixing bacteria and phosphate-solubilizing bacteria and plants are unknown.

This study showed that when two species of these bacteria, isolated from the same tree, are mixed in vitro, in either culture medium or seawater, they affect each other's metabolism. N_2-fixation increased in the N_2-fixing bacterium, *Phyllobacterium* sp., and phosphate solubilization increased in *B. licheniformis*. The effect of the mixed inoculation on black mangrove seedlings was moderate. The average number of true leaves in the seedlings increased as did the incorporation of ^{15}N into the leaves and stem tissues. This indicates a direct transfer of nitrogen from the N_2-fixing bacterium to the plant, similar to that which occurred when the diazotrophic cyanobacterium *M. chthonoplastes* was used to inoculated black mangrove seedlings (6,19).

In sum, this study showed that marine bacterial species, with a potential as plant growth-promoting bacteria interact when mixed.

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