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Synergism between *Phyllobacterium* sp. (N_2-fixer) and *Bacillus licheniformis* (P-solubilizer), both from a semiarid mangrove rhizosphere

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Abstract

Mangrove seedlings were treated with a mixture of two bacterial species, the slow-growing, N_2-fixing bacterium *Phyllobacterium* sp. and the fast-growing, phosphate-solubilizing bacterium *Bacillus licheniformis*, both isolated from the rhizosphere from black, white, and red mangroves of a semiarid zone. Nitrogen fixation and phosphate solubilization increased when the mixture was used compared to the effects observed when adding individual cultures, notwithstanding that there was no increase in bacterial multiplication under these conditions. Inoculation of black mangrove seedlings in artificial seawater showed the mixture performed somewhat better than inoculation of the individual bacterium; more leaves were developed and higher levels of ^{15}N were incorporated into the leaves, although the total nitrogen level decreased. This study demonstrates that interactions between individual components of the rhizosphere of mangroves should be considered when evaluating these bacteria as plant growth promoters.

Keywords: Mangrove; Mutualism; Nitrogen fixation; Phosphate solubilization; Plant growth-promoting bacterium; *Phyllobacterium*; *Bacillus licheniformis*

1. Introduction

Sediment and rhizosphere microorganisms are the major biological components that contribute to the productivity of mangroves [1–6]. Because of diverse microbial activity, mangrove ecosystems are one of the three most productive ecosystems known, along with rain forests and coral reefs [7]. Yet, mangroves are alarmingly and systematically being deforested, similarly to rain forests [8,9]. To aid in the reforestation of mangroves, inoculation of the seedlings with plant growth-promoting bacteria (PGPBs) has been suggested [10,11,12], similarly to what has been done in agriculture [13–16] and temperate forestry [17].

At the present time, little is known about the PGPBs and their mechanisms and interactions that are normally associated with mangroves. Previous work on PGPBs that interact with mangroves includes the observation that the diazotrophic cyanobacteria *Microcoleus chthonoplastes* improved N_2 fixation [12] and nitrogen incorporation in black mangrove seedlings [10]. In addition, the terrestrial halotolerant bacterium *Azospirillum halopraeferens* [18] and halotolerant *Azospirillum brasilense* Cd [19] can successfully colonize black mangrove roots in seawater [11]. More recently, several potential PGPBs of mangrove origin (i.e. *Bacillus licheniformis*, *M. chthonoplastes*, *Phyllobacterium myrsinacearum*, *Vibrio aestuarianus*, and *Vibrio proteolyticus* [12,20]) were shown to promote the growth of the annual, potential oilseed seaweed *Salicornia bigelovii*, which shares the semiarid mangrove ecosystem with the trees [21].

In agricultural and forestry inoculation practices, mixing of two or more microbial species often has a more positive effect on plant growth than the use of a single bacterium [22–28].

The interaction of N_2-fixing bacteria with other bacteria can inhibit or promote their diazotrophic activity [29,30]. The degradation of cellulose by *Cellulomonas* sp. provided *Azospirillum* sp. with a usable carbon source to obtain energy for N_2 fixation. The contribution of *Azospirillum* to *Cellulomonas* is fixed nitrogen [31]. The association between different *Azospirillum* species and N_2-fixer *Bacillus polymyxa* enhanced the N_2-fixing activity of the mixed

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culture compared to the pure culture of each bacterium. *Azospirillum* benefited from the products that are released from the degradation of pectin by *B. polymyxa* [32]. The N_2-fixing activity of *A. brasilense* Cd increased significantly when grown in mixed culture with the mangrove rhizosphere bacterium *Staphylococcus* sp., releasing aspartic and succinic acids into the growth medium [19]. Apart from the increase in N_2 fixation when the mangrove N_2-fixer *Listonella anguillarum* was grown together with *Staphylococcus* sp. [33], other interactions of potential mangrove PGPBs among themselves and their effect on plant growth are unknown.

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

2. Materials and methods

2.1. Organisms and growth conditions

Phyllobacterium sp. was isolated according to Holguin et al. [33] from the rhizosphere of semiarid black (*Avicennia germinans*), white (*Laguncularia racemosa*), and red (*Rhizophora mangle*) mangrove seedlings from Laguna de Balandra, Baja California Sur, Mexico. Its nitrogen-fixing capacity was assessed as described by Holguin et al. [33]. This bacterium was characterized using both fatty acid methyl ester analysis and 16S rRNA by a commercial service (Acculab, Newark, DE, USA). Although the analyses indicate this bacteria is probably *Phyllobacterium rubiacearum* or *P. myrsinacearum*, it did not produce nodules on leaves, fix atmospheric nitrogen, and its habitat is seawater. These characteristics do not fit the species description, but it did show star clusters, and its colonies and cellular characteristics do fit the genus description [34,35]. Therefore, we chose to cite this strain as *Phyllobacterium* sp. *B. licheniformis*, originally isolated from black and white mangroves with a phosphate solubilization capacity of 325 mg P l^{-1}, was obtained from the CIB culture collection. It was purified and characterized in a previous study [20]. Both of the above bacterial species were maintained for routine use in 30% glycerol (v/v) and stored in modified SRSM2 medium for *B. licheniformis* [20] and N-free medium for *Phyllobacterium* sp. at either -40 ± 3°C or in liquid nitrogen.

Black mangrove propagules were collected from Laguna de Balandra, Baja California Sur, Mexico [36,37]. They were treated and inoculated with the bacteria as previously described [10,12].

All experiments were done in vitro. Bacteria were grown in unbaffled Erlenmeyer flasks on an environmentally controlled rotary shaker at 150 rpm (*B. licheniformis*), or without agitation (*Phyllobacterium* sp.) at 30 ± 1°C. Three culture media were used; (i) both liquid and solid minimal medium SRSM2 [20] supplemented with 1.1 g $K_2HPO_4 \cdot 3H_2O$ l^{-1} (Sigma), (ii) N-free minimal medium for marine bacteria containing (g l^{-1}): 0.075, $K_2HPO_4 \cdot 3H_2O$; 0.028, $FeSO_4 \cdot 7H_2O$; 1.25, malic acid; 1.25, citric acid; 2, D-glucose; 2, D-mannitol; 1, myo-inositol; 23.4, NaCl; 1.5, KCl; 24, $MgSO_4 \cdot 7H_2O$; 2.9, $CaCl_2 \cdot 2H_2O$; 10 ml glycerol; (μg l^{-1}) 0.01, biotin; 0.02, pyridoxine; 0.4, $Na_2MoO_4 \cdot 2H_2O$; 4.7, $MnSO_4 \cdot H_2O$; 0.56, H_3BO_3; 0.16, $CuSO_4 \cdot 5H_2O$; 0.048, $ZnSO_4 \cdot 7H_2O$ (Holguin, G., 1997; Technical report for Consejo Nacional de Ciencia y Tecnologia, Mexico), and (iii) filtered seawater [11]. Incubation times varied according to the experiment; up to 5 days for phosphate and nitrogen fixation tests, 5 days for cultures containing mangrove seedlings for acetylene-reduction assay, and 26 days for cultures containing mangrove seedlings (7 cm tall) for total nitrogen content and ^{15}N abundance.

Bacterial counts were done by the Plate Count Method [38] on Nutrient Broth medium (Difco) supplemented with 2% NaCl.

2.2. Analyses

Nitrogen fixation was measured by the acetylene-reduction assay using gas chromatography after 48 h of mixed incubation as shorter periods showed negligible values [33]. Root surface area was measured using the calcium nitrate gravimetric method [39]. Total nitrogen was measured by an automatic micro-Kjeldahl procedure after digestion (Digestion System 12.1009, and Kjeltec Auto 103 analyzer, Tecator, Höganäs, Sweden). Abundance of ^{15}N in the sample was measured by Isochrom Continuous Flow, Stable Isotope Mass Spectrometer (Micromass, Manchester, UK) according to standard methods [40] and expressed as ^{15}N in parts per thousand (δ) as previously described [10]. Phosphate solubilization was done using a modification of the method of Vazquez et al. [20] as follows: *B. licheniformis* was grown on SRSM2 solid medium for 24 h and *Phyllobacterium* sp. for 120 h on N-free medium. All colonies were aseptically scraped from the agar surface, each suspended in 1 ml of 2% NaCl, and the number of colony-forming units (cfu) ml^{-1} determined. The two cultures were then mixed and inoculated into 25 ml of natural seawater [11] supplemented with 5 g l^{-1} tribasic calcium phosphate (Sigma) and incubated under agitation of 150 rpm at 28°C for an additional 120 h. Three-ml samples from this culture were centrifuged at $14\,000 \times g$ for 3 min. The supernatant (2.5-ml samples) was collected and diluted to 25 ml with distilled water. Phosphate analysis was done according to standard methods of water analysis [41] as modified by the Hach Co. (CO, USA) using a Hach DR2000 spectrophotometer (Hach program # 480) at 430 nm. Light microscopy of freshly mounted cultures and spore staining (methylene blue) were done by using a Zeiss (Germany) light microscope ($\times 1000$).

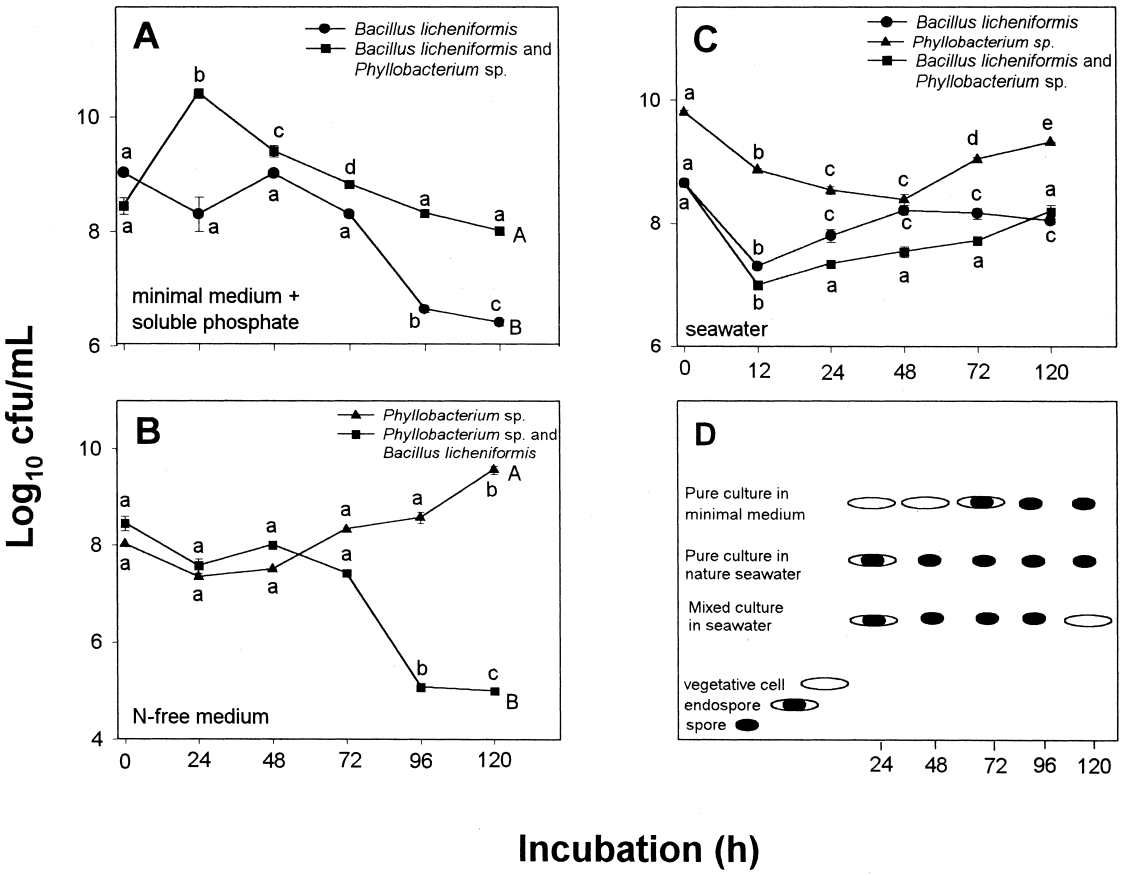

Fig. 1. Growth curves of *Phyllobacterium* sp. and *B. licheniformis* in pure and mixed cultures. (A) Minimal medium SRSM2, (B) N-free minimal medium, (C) seawater. Points denoted by a different lower case letter for each bacterial growth curve separately differ significantly at $P \leq 0.05$ by one-way ANOVA. Points in each sampling time denoted by different capital letter in each subfigure separately differ significantly at $P \leq 0.05$ by the Student's *t*-test. Bars represent S.E.M. The absence of S.E.M. means that it is smaller than the point. (D) Appearance and disappearance of spores of *B. licheniformis* during growth in different culture media.

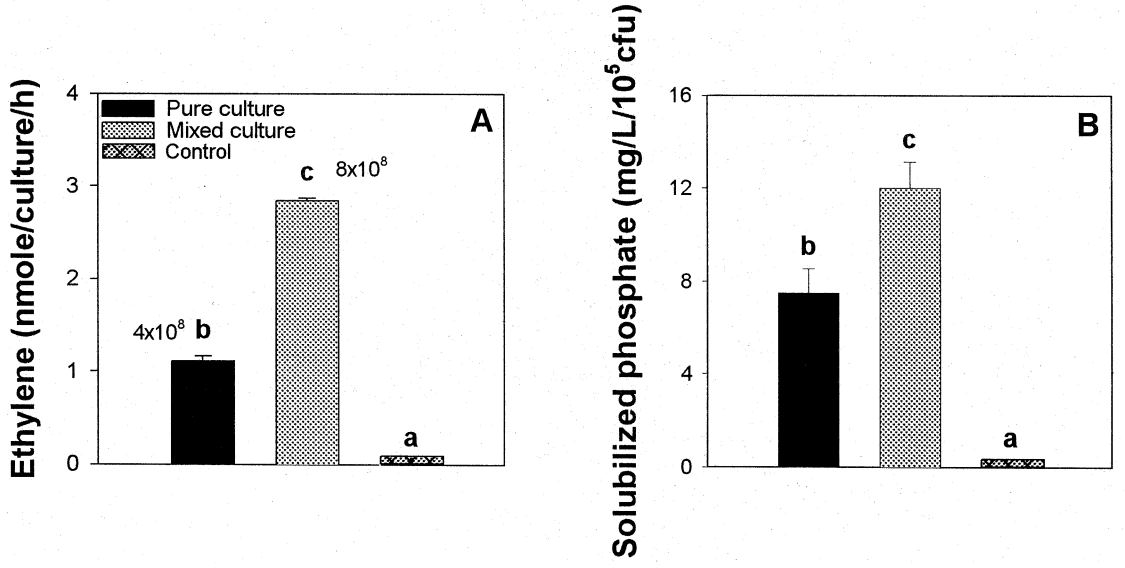

Fig. 2. (A) Nitrogen fixation (acetylene reduction) of bacterial cultures inoculated with the N_2-fixing bacteria *Phyllobacterium* sp. alone or mixed with the phosphate-solubilizing bacteria *B. licheniformis*. (B) Phosphate solubilization of *B. licheniformis* cultures alone or together with *Phyllobacterium* sp. Columns denoted by a different letter, in each subfigure, differ significantly at $P \leq 0.05$ by one-way ANOVA. Number above each column represents the bacterial population in cfu ml^{-1}. Bars represent S.E.M. Absence of S.E.M. indicates minimal S.E.M.

2.3. Experimental design and statistical analysis

All experiments were done in 10 replicates using an Erlenmeyer flask (for bacteria) or filtration flask containing one mangrove seedling as a replicate. All experiments were repeated 2–3 times. Analysis of ^{15}N was done once. Results were analyzed using one-way Analysis of Variance (ANOVA) or by the Student's *t*-test, both at $P \leq 0.05$, using Statistica software (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Growth of *Phyllobacterium* sp. and *B. licheniformis* in mixed cultures

Light microscopy revealed that when the two bacterial species were grown on solid medium they formed one morphotype colony containing both species, whereas on liquid medium they grew as separate cells. Thus, the counts on solid medium of mixed cultures done in this study could not distinguish between the different populations.

On minimal medium having a soluble P source, the fast grower *B. licheniformis* maintained its population in the first 72 h, then gradually the population decreased. The mixed population increased bacterial population in the first 24 h, and then decreased (Fig. 1A). *Phyllobacterium* sp., a slow grower, continued its growth for 120 h when grown alone in N-free medium (having a soluble P source). In mixed culture on this medium, a similar decrease in population occurred as with the previous medium (Fig. 1B). In seawater, after an initial decrease in their populations, both bacterial species increased their population over time as did the mixed population, approximately to their initial level. The decrease in the mixed population observed in the previous two media was not apparent (Fig. 1C).

Phyllobacterium sp. cells did not change their morphology in culture whether alone or in a mixture. However,

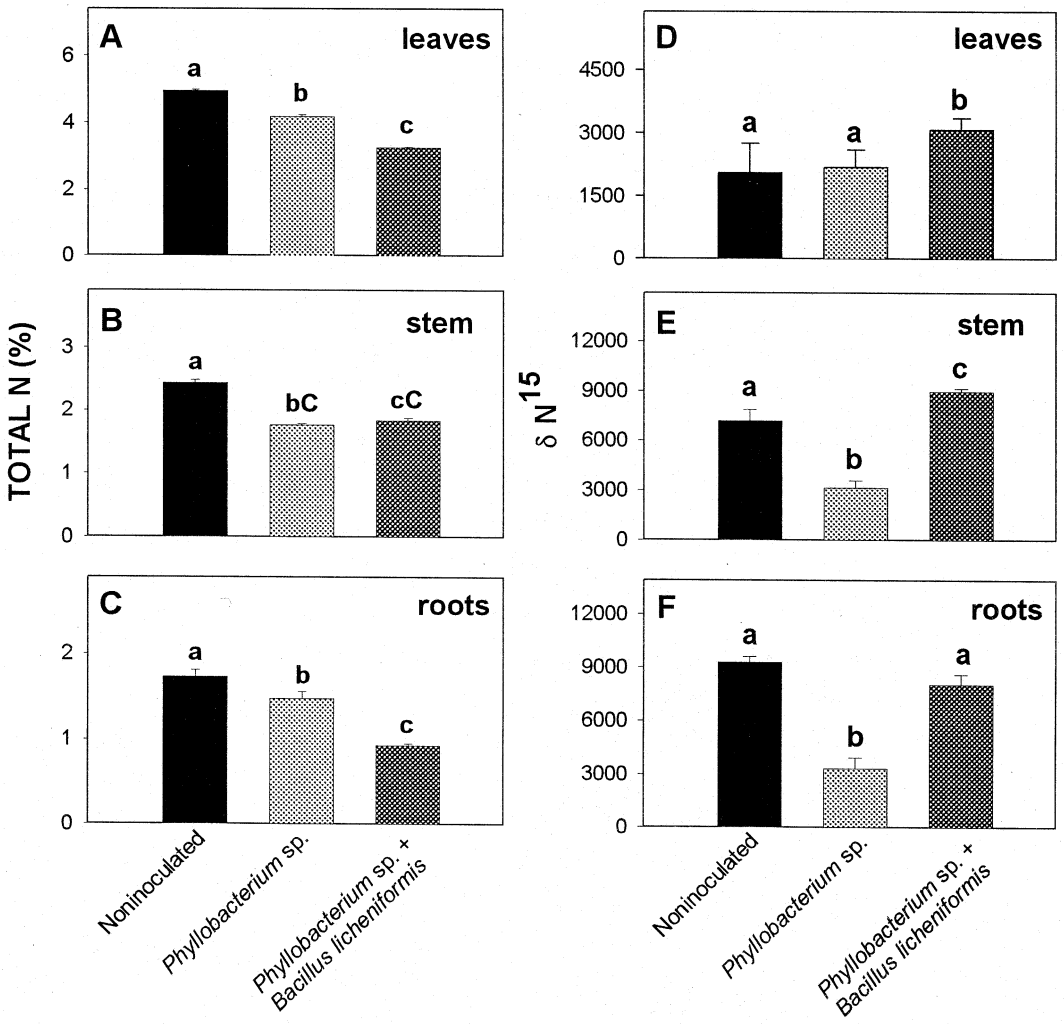

Fig. 3. Total nitrogen content of black mangrove seedlings inoculated with the N_2-fixing bacteria *Phyllobacterium* sp. alone or mixed with the phosphate-solubilizing bacteria *B. licheniformis* (A–C). ^{15}N accumulation in the same plants (D–F). Columns denoted by a different letter, in each subfigure, differ significantly at $P \leq 0.05$ by one-way ANOVA. Bars represent S.E.M. Absence of a bar above a column indicates minimal S.E.M.

cells of the fast-growing *B. licheniformis* produced spores that appeared at different incubation times depending on the medium. In pure culture in minimal medium, endospores were observed after 72 h of incubation. In seawater, endospores were found within the first 24 h, and older cultures consisted almost exclusively of spores. A mixed culture in seawater resembled pure cultures in seawater. First there was spore formation but different from the pure culture. After 120 h there was massive reappearance of vegetative cells (Fig. 1D).

3.2. Nitrogen fixation, phosphate solubilization, and nitrogen accumulation in seedlings inoculated with mixed cultures

Acetylene reduction of mixed cultures revealed that *Phyllobacterium* sp. fixes twice the amount of nitrogen than when it is in pure culture (Fig. 2A). Similarly, phosphate solubilization by *B. licheniformis* increased significantly in mixed as compared to monocultures (Fig. 2B). Black mangrove seedlings inoculated with either pure or mixed cultures showed similar levels of nitrogen fixation (10.7 nmol ethylene per plant per 7 days). All inoculation treatments reduced the nitrogen content in the leaves, stems, and roots of the plants (Fig. 3A–C). The ^{15}N content in the plant leaves and stems was significantly higher in mixed culture than in uninoculated plants (Fig. 3D–F).

3.3. Root colonization and the effect of inoculation on black mangrove seedlings

All inoculations significantly increased the number of true leaves of black mangrove seedlings over the uninoculated control; however, there was no significant difference among the inoculation treatments (Fig. 4). None of the inoculations had any effect on the root surface area of the seedlings. Root colonization of the seedlings was at a level of 2×10^4 cfu g^{-1} dw. root for both pure cultures and 1×10^5 cfu g^{-1} dw. root for the mixed culture. We could not differentiate between the two bacterial species as they grew as one mixed colony on the root surface.

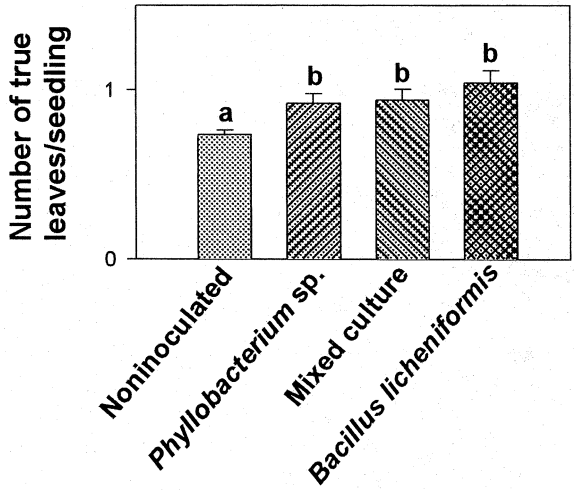

Fig. 4. Effect of inoculation with the N_2-fixing bacteria *Phyllobacterium* sp. and the phosphate-solubilizing bacteria *B. licheniformis* alone or together on the number of true leaves developed by the seedlings. Columns denoted by a different letter differ significantly at $P \leq 0.05$ by one-way ANOVA. Bars represent S.E.M.

4. Discussion

Semiarid mangrove forests thrive in lagoons that are lacking sources of both dissolved phosphorus and nitrogen [5,20], essential growth elements for any plant species. Nitrogen fixation is a well documented phenomenon that must be present in any mangrove ecosystem [42–47]. Several species of N_2-fixing mangrove root-associated bacteria were isolated and identified [33,37].

Many species of microorganisms can solubilize phosphate under marine environments. Phosphate-solubilizing bacteria can be found in the water column [48], in the rhizosphere of the seaweed *Zostera marina* [49], and associated with white and black mangroves [20]. Their number can be as high as 10^7 cfu g^{-1} sediment [50]. At present, the interactions, if any, between mangrove root-associated N_2-fixing bacteria and phosphate-solubilizing bacteria and plants are unknown.

We showed that when two species of mangrove-associated bacteria are mixed in vitro in a culture medium or in seawater, they affect each other's metabolism. When grown in culture medium, the two populations, after keeping a steady state for 24 and 72 h, declined probably because of nutrient exhaustion. When the mixture was grown in seawater, this did not happen and the mixture then had an advantage over single cultures. Although one may expect nutrient exhaustion to be more apparent in seawater, seawater would appear to be a better culture medium for this marine diazotrophic bacteria than the N-free medium used in his study.

The two bacterial species, originally isolated from the same tree, mutually affect one another's metabolism in mixed cultures in vitro. Nitrogen fixation increased in the N_2-fixing bacterium *Phyllobacterium* sp., and phosphate solubilization increased in *B. licheniformis*. The effect of 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 inoculated on black mangrove seedlings [10,12]. However, the total nitrogen content of the plant decreased. We might speculate that the supply of nitrogen by the N_2-fixing bacterium was insufficient to keep pace with the increased growth of the seedling (from 9.5 to 10 cm tall in 26 days) and to

supply the requirements of the phosphate-solubilizing *B. licheniformis*.

In sum, we showed that bacterial species, each with a potential as a PGPB, can interact when mixed and their metabolism is affected. Though the black mangrove rhizosphere and aerial roots are heavily colonized by numerous bacteria and other protista [37], it is premature to speculate whether the effects that were detected also occur in the rhizosphere, and if so at what magnitude.

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