Isolation of *Atriplex nummularia*-associated halotolerant bacteria and bioprospecting by nitrogen fixing bacteria in saline-sodic soil

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Abstract
The plant-associated habitat is a dynamic environment exploited by a wide variety of bacteria. These bacteria can contribute to the health, growth and development of plants by different mechanisms. *Atriplex* plants cultivated in saline sodic soils stimulated a bioprospection of bacteria-*Atriplex* interaction mechanisms. Therefore, the aim of this work was to study the interaction *Atriplex nummularia*- halotolerant bacteria and bioprospect nitrogen fixing bacteria. Leaf and root endophytic bacteria and bacteria from rhizosphere, cultivated soil and uncultivated soil were isolated using 10% TSA plus 50 g l^{-1} of NaCl. Furthermore, the screening for nitrogen fixing bacteria was evaluated by the ability to grow in semi-solid nitrogen-free NFb medium. The numbers of total cultivable halotolerant bacteria were significant between leaves, roots and rhizoplane of *A. nummularia* plants. The NFb medium methodology revealed that 56% of analyzed halotolerant isolates were able to grow in nitrogen free medium. The percentage of nitrogen fixing bacteria was higher for the bacteria in close interaction with the host plant than for the less related niches. The results from this study indicated that *Atriplex nummularia*-associated halotolerant bacteria are able to fix nitrogen and were found at higher percentage in close interaction with the host plant.

Key Words
Salinity, endophytic bacteria, interaction bacteria-plant, plant growth promotion.

Introduction
Plants may be considered to be a complex microecosystem where different niches are exploited by a wide variety of bacteria. Such niches include not only the external surfaces of plants, but also the internal tissues into which endophytic bacteria inhabit the interior of plants showing no apparently harm to the host or external structures (Azevedo *et al.* 2002). The plant-associated habitat is a dynamic environment in which many factors may affect the structure and species composition of the bacterial communities that colonize plant tissues. Some of these factors are seasonal changes, plant tissue (Mocali *et al*. 2003; Kuklinsky-Sobral *et al.* 2004), plant species and cultivar, soil type (Fromin *et al*. 2001; Kuklinsky-Sobral *et al*. 2004) and interaction with other beneficial or pathogenic microorganisms (Araújo *et al*. 2002; Lacava *et al*. 2007). Soil and plant-associated bacteria can contribute to the health, growth and development of plants by different mechanisms, such nitrogen fixing or production of phytohormones (Rosenblueth and Martinez-Romero 2006). The *Atriplex* spp. are salt tolerant, producers of biomass with high content of crude protein and are used as forage fodder shrubs (Bilal *et al.* 1990). These plants cultivated in low-fertility saline sodic soils stimulated a bioprospection of bacteria-*Atriplex* interaction mechanisms. Therefore, the aim of this work was to study the interaction *Atriplex nummularia*- halotolerant bacteria and bioprospect nitrogen fixing bacteria.

Methods
Plant and experimental field design
A field experiment was made with *Atriplex nummularia* cultivated in a saline-sodic soil (CE = 42.56 dS m^{-1}; PST = 71.20%) at Pernambuco State, Brazil (8º 34' 17" South and 37º 1' 20" West) during one year in a randomized block design, without irrigation. The soil was cultivated with plants in two treatments (with and without cutting at six months) and an uncultivated soil treatment, in four replicates.

Isolation of soil and Atriplex nummularia associated bacteria
Atriplex nummularia plants, rhizosphere, cultivated soil and uncultivated soil (0–0.2m layer) samples were collected and immediately transported to the laboratory where the plants were washed in running tap water to remove soil and the leaves and roots were separated.

Bacteria from rhizosphere, cultivated soil and uncultivated soil were isolated by placing five grams of soil in a 500 ml erlenmeyer flask containing 25 g of 0.1 cm diameter glass beads and 50 ml of phosphate buffered saline [PBS, containing (g l^{-1}) Na_2HPO_4, 1.44; KH_2PO_4, 0.24; KCl, 0.20; NaCl 8.00; pH 7.4] and agitating the flasks at 100 rpm, 28°C for 1 h. Rhizoplane bacteria were isolated by placing three grams of root tissue in a 500 ml erlenmeyer flask containing 25 g of 0.1 cm diameter glass beads and 50 ml of PBS and agitating the flasks at 100 rpm, 28°C for 1 h. After agitation, appropriate dilutions of the contents of the flasks were plated onto 10% trypicase soy agar (TSA) plus 50 g l^{-1} of NaCl and supplemented with 50 μg ml^{-1} of the fungicide Thiophanate methyl (Cercobin 700 PM, DuPont) and the plates incubated at 28°C for 2 to 15 days, after which colonies were picked off the plates, inoculated on 10% TSA agar slants, incubated at 28°C for 2 days and stored at 4°C. These colonies also were cultivated in 10% TSA, incubated at 28°C for 18 hours and following each culture was suspended in 20% glycerol solution and stored at -20°C.

Leaf and root endophytic bacteria were isolated according to Kuklinsky-Sobral *et al.* (2004) with some modifications. The surface disinfection process was done using serial washing in 70% ethanol for 1 min, sodium hypochlorite solution (2% available Cl^-) for 3 min, 70% ethanol for 30s and two rinses in sterilized distilled water. The disinfection process was checked by plating aliquots of the sterile distilled water used in the final rinse onto 10% TSA supplemented with 50 μg ml^{-1} of the fungicide Thiophanate methyl and incubating the plates at 28°C for 2 to 15 days. After surface disinfection, the leaf and root tissue was cut and triturated in 10 ml of sterile PBS contained in a 50 ml flask maintained at 28°C and agitated at 150 rpm for 1 h, after which appropriate dilutions were plated onto 10% TSA plus 50 g l^{-1} of NaCl and supplemented with 50 μg ml^{-1} of the fungicide Thiophanate methyl and incubated at 28°C for 2 to 14 days. After incubation, colonies were picked off the plates, inoculated on 10% TSA agar slants, incubated at 28°C for 2 days and stored at 4°C. These colonies also were cultivated in 10% TSA, incubated at 28°C for 18 hours and following each culture was suspended in 20% glycerol solution and stored at -20°C.

Screening for nitrogen fixing bacteria
The screening for nitrogen fixing bacteria was evaluated by the ability to grow in semi-solid nitrogen-free NFb medium (Dobereiner *et al.* 1995); a halo of bacterial growth within the medium indicates nitrogen fixation.

Results and Discussion
The interaction between halotolerant bacteria and *Atriplex nummularia* plants was assessed in leaves, roots, rhizoplane, rhizosphere, cultivated soil and uncultivated soil. Several bacterial morphotypes were observed on 10% TSA medium plus NaCl 50g l^{-1} (Figure 1). The number of total cultivable halotolerant bacteria was not significantly different among the soil treatments and rhizosphere, but was significant between leaves, roots and rhizoplane of *A. nummularia* plants (Figure 2). The plant-associated habitat is a dynamic environment in which many factors may affect the structure and species composition of the bacterial communities that colonize plant tissues. Some of these factors are seasonal changes, plant tissue, plant species and cultivar, soil type (Fromin *et al.* 2001; Mocali *et al.* 2003; Kuklinsky-Sobral *et al.* 2004). An understanding of the structure and species composition of plant-associated bacterial populations is fundamental to understanding how plant-associated biological processes are influenced by environmental factors and, consequently, has important biotechnological implications.

A total of 41 *A. nummularia*-associated halotolerant bacteria isolated from leaves, roots, rhizoplane, rhizosphere, cultivated soil and uncultivated soil were randomly picked up and were evaluated for their possible ability to fix atmospheric nitrogen. The methodology used was bacterial growth in nitrogen free medium (NFb medium), a halo of bacterial growth within the medium revealed nitrogen fixation (Figure 3). The NFb medium methodology revealed that 56% of analyzed halotolerant isolates were able to grow in nitrogen free medium. In this context, Bilal *et al.* (1990) observed root-associated nitrogenase activity in *Atriplex* spp. However the percentage of nitrogen fixing bacteria was higher (more than 60%) for the bacteria in close interaction with the host plant than for the less related niches (Figure 4). As described previously (Elvira-Recuenco and van Vuurde, 2000) the roots seem to be the preferential site for epiphytic and endophytic bacteria, suggesting that endophytic bacteria may travel upward from the roots into the stem during plant development.

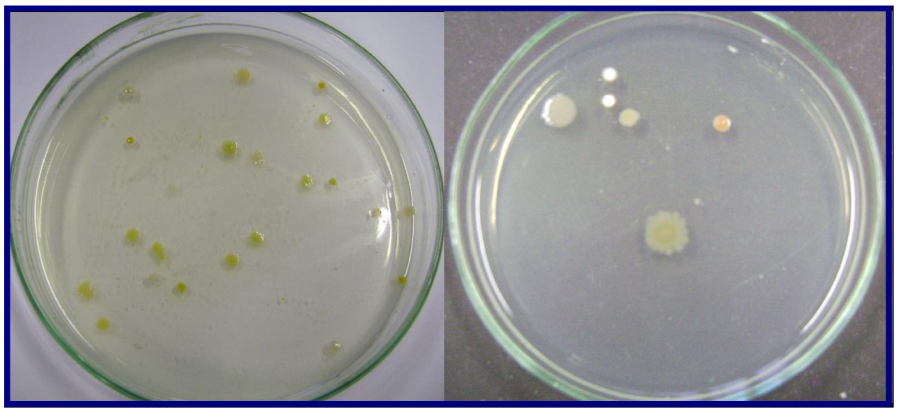

Figure 1. Morphotypes of *Atriplex nummularia*-associated halotolerant bacteria.

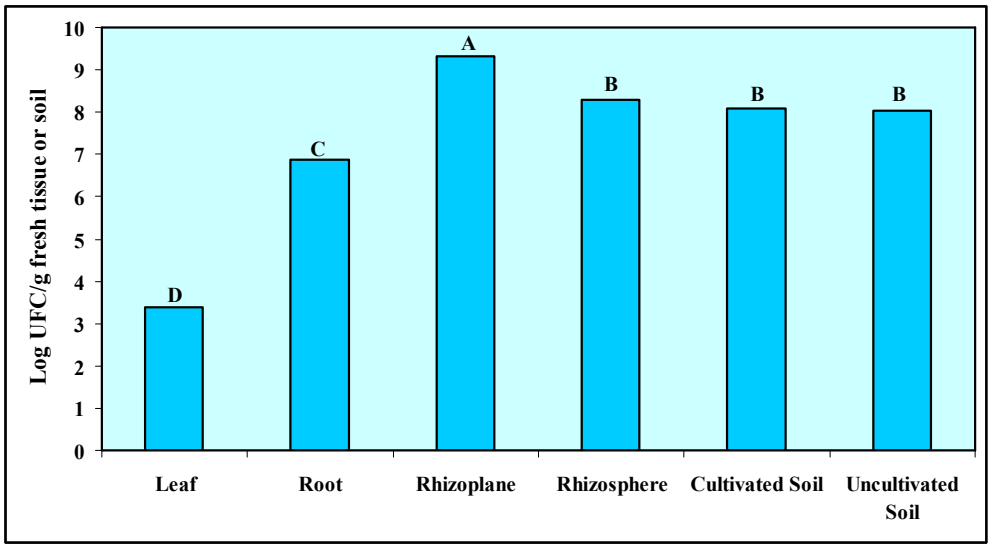

Figure 2. Total population density of *A. nummularia*-associated halotolerant bacteria isolated from: leaf, root, rhizoplane, rhizosphere, cultivated soil and uncultivated soil. Means with the different letters are significantly different by the Tukey test (P<0.05).

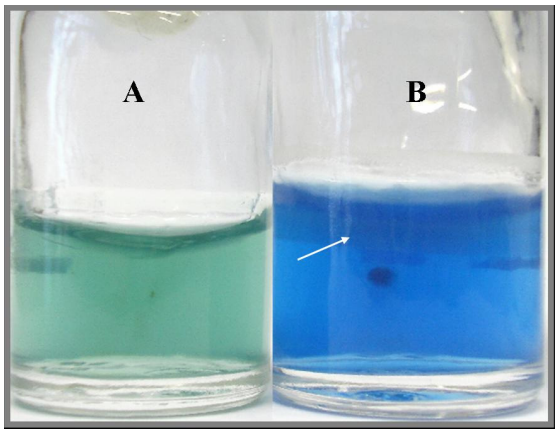

Figure 3. Negative (A) and positive (B) reaction to the test of biological nitrogen fixation in NFb medium. The arrow indicates the halo of bacterial growth.

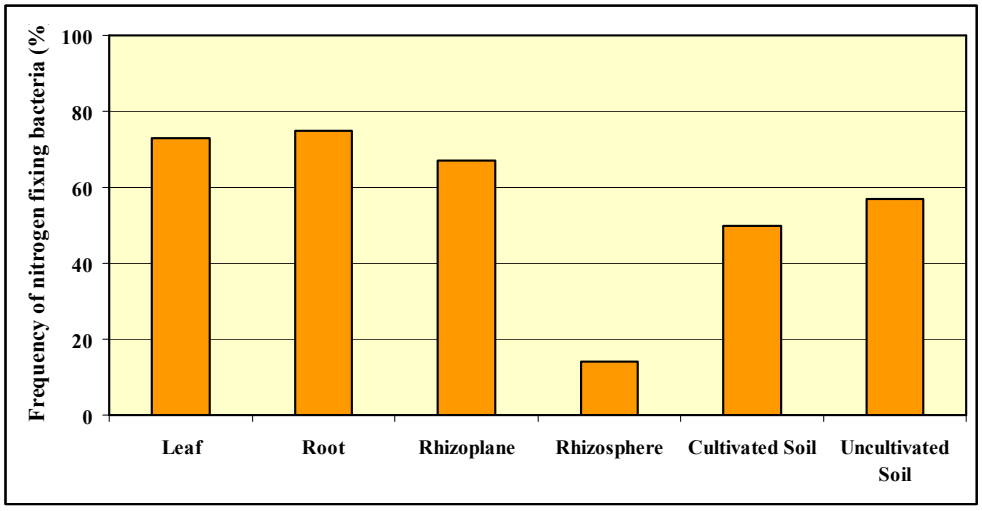

Figure 4. Frequency of nitrogen fixing *A. nummularia*-associated bacteria isolated from: leaf, root, rhizoplane, rhizosphere, cultivated soil and uncultivated soil.

Conclusion

The results from this study indicated that *Atriplex nummularia*-associated halotolerant bacteria are able to fix nitrogen and were found at higher percentage in close interaction with the host plant. However, a more complete comprehension of the interaction between *A. nummularia* and associated halotolerant bacterial communities is an important factor for a more effective crop management and should be further evaluated in field experiments.

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