ENVIRONMENTAL APPLICATIONS FOR *AZOSPIRILLUM* Sp.

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Plant growth-promoting bacteria (PGPB) of the genus *Azospirillum* are commonly used to improve agricultural plant yields. In addition to their usefulness as an agricultural inoculant, their potential benefits can be extended to environmental applications. For example, *Azospirillum* species can enhance the bioremediation of wastewater by microalgae by increasing algal proliferation and metabolism. *Azospirillum* species may improve the reforestation of marine mangrove trees, thereby enhancing fisheries along tropical coasts and may prevent desert soil erosion and promote abatement of dust pollution by assisting in the growth of cactus species. The following minireview discusses these applications, and shows future potential avenues for *Azospirillum* as an environmentally friendly microorganism.

Wastewater bioremediation. Microalgae have many uses, including water bioremediation. For such use, it is usually desirable to establish large populations of microalgae in aquatic environments. One means of increasing microalgal populations may be to inoculate them with PGPBs. A candidate microorganism for coinoculation with microalgae is *Azospirillum brasilense* (strain Cd), a known plant growth-promoting bacterium. To improve the growth, metabolism, and removal of nitrogen and phosphorus by the freshwater microalga *Chlorella vulgaris* (UTEX 2714), an important organism often used in wastewater treatment, *C. vulgaris* was inoculated with *A. brasilense.* The two microorganisms were kept in close proximity in the liquid medium essential for *C. vulgaris* by coimmobilization in alginate beads and were cocultivated under controlled conditions suitable for both, in batch cultures and in continuous flow cultures in a chemostat. Alginate beads of various forms and shapes are convenient inoculant carriers for use in numerous industrial, environmental, and agricultural applications.

Coimmobilization of the freshwater microalga *C. vulgaris* and *A. brasilense* in small alginate beads resulted in significant increased growth of the microalga. Dry and fresh weight, total number of cells, size of the microalgal clusters (colonies) within the bead, number of microalgal cells per cluster, and the levels of microalgal pigments significantly increased. Light and transmission electron microscopy revealed that both microorganisms colonized the same cavities inside the beads, though the microalgae tended to concentrate in the more aerated periphery, and the bacteria colonized the entire bead. The effect of indole-3-acetic acid (IAA) addition to the microalgal culture prior to immobilization of microorganisms in alginate beads partially imitated the effect of *A. brasilense*.

Coimmobilization of *C. vulgaris* UTEX 395 and *C. sorokiniana* with *A. brasilense* resulted in significant changes in the microalgae cell morphology and pigment content. The size of *C. sorokiniana* cells not changed; however, the population within the beads significantly increased. In contrast, *C. vulgaris* UTEX 395 cells grew larger but their number did not increase. Similar to *C. vulgaris* UTEX 2714, the pigment content of the microalgal cells significantly increased as a result of coimmobilization**.**

The ability of the coimmobilized culture to clean wastewater (to remove ammonium and phosphorus from the water) was analyzed in continuous cultures and in step cultures where the wastewater was replaced every 48 hours. In continuous cultures, only moderate levels were removed. However, in step cultures almost all of the ammonium was removed. After six consecutive 48 hour cycles, the bioremediation system was saturated and the ammonium removal efficiency decreased. In comparison, saturation was reached after 3 cycles with immobilized microalgae alone and the level of ammonium removal was reduced.

In another study, *C. vulgaris* (UTEX 2714) was coimmobilized in alginate beads and coincubated with either *A. brasilense,* or with its natural associative bacterium *Phyllobacterium myrsinacearum*. The interactions between the microalga and the bacterial species were followed by transmission electron microscopy for 10 days. Most of the small cavities within the beads were colonized by microcolonies of only one microorganism regardless of the bacterial species cocultured with the microalga. Subsequently, the bacterial and microalgal microcolonies merged to form large, mixed colonies within the cavities. At this stage, the effect of bacterial association with the microalga differed depending on the bacterium present. The microalga entered a senescence phase in the presence of *P. myrsinacearum*, but remained in a growth phase in the presence of *A. brasilense.* This study suggests that there are commensal interactions between the microalga and the two plant associative bacteria and that with time the bacterial species determines whether the outcome for the microalga is senescence or continuous multiplication.

The deliberate inoculation of *Chlorella* sp. with a terrestrial PGPB was not reported prior to these studies, perhaps because of the different origins of the two microorganisms. *C. vulgaris* is not known to harbor any associative beneficial bacteria, and *Azospirillum* sp. is rarely used for inoculation in aquatic environments. These studies indicate that the changes induced in the microalga by the plant growth-promoting bacterium may improve the potential of the microalga as a wastewater treatment agent (7,8). We propose that coimmobilization of microalgae and plant growth-promoting bacteria are an effective means of increasing microalgal populations within confined environments and of improving wastewater-cleaning capacity.

Mangrove reforestation. Mangroves are tropical and subtropical trees that are the major component of coastal marine-lagoon ecosystems and serve as feeding, spawning, and reproductive areas for numerous economically and ecologically important marine species. Tropical mangroves generally revegetate themselves after clearcutting. However, in semiarid tropics, after clearcutting, mangroves rarely revegetate. In addition, mangrove ecosystems are considered to be nutrient-deficient because they contain low levels of nitrogen and phosphorous. Despite the low levels of nutrients, these ecosystems flourish with no obvious signs of nutrient deficiency. Sediment and rhizosphere microorganisms are the major biological components that assure mangrove productivity. Probably because of microbial activity, mangrove ecosystems are one of the three most productive ecosystems on a global scale; the other two highly productive ecosystems are rain forests and coral reefs.

Despite their importance, mangroves face the same destructive deforestation as the rain forests. To aid mangrove reforestation, it has been proposed that seedlings are inoculated with plant growth-promoting bacteria (PGPB), a practice that has been successful in agriculture and temperate forestry. Mangrove seedlings usually grow better after inoculation with the diazotrophic filamentous cyanobacteria M*icrocoleus chthonoplastes* (3). Based on this observation, it was reasoned that mangrove seedlings might also benefit from inoculation with plant growth-promoting bacteria. Nitrogen fixation by inoculants in mangrove sediments, in the rhizosphere, and associated with aerial roots may provide the nitrogen necessary for plant growth, and phosphate-solubilizing microorganisms may supply plants with a sufficient amount of phosphorus.

Inoculation of axenic black mangrove seedlings in seawater for eight days with either the terrestrial halotolerant plant growth-promoting bacterium *A. halopraeferens* or with *A. brasilense* produced heavy colonization of the root surface. The colonization pattern was different for the two strains. *A. halopraeferens* was present mainly as single cells embedded in a thick sheath, whereas *A. brasilense* produced primarily microaggregates. *A. brasilense* cells were anchored to the root surfaces and to each other by a network of fibrillar material. Both bacterial strains survived in seawater (approx. 10^4 cfu/mL) for more than 30 days, and colonized mangrove roots at a high density. While *A. halopraeferens* was a better root surface colonizer, *A. brasilense* was better able to populate the entire root (surface and inside) (9).

Plant growth-promoting bacteria native to mangrove ecosystems are almost unknown. Recently, several bacteria isolated from mangroves promoted the growth of *Salicornia bigelovii*, a potential oilseed crop that grows in semiarid mangrove areas (2).

In agricultural and forestry practices, inoculation of plants with a mixture of two or more microbial species often has a greater effect on plant growth than inoculation with a single strain. The interactions among potential mangrove PGPBs and the effect of microbial mixtures on mangroves have not yet been explored. Two potential mangrove PGPBs obtained, from the rhizosphere of semiarid zone mangroves, were mixed in vitro in nitrogen-free culture medium or in seawater; the slow-growing N_2-fixing bacterium *Phyllobacterium* sp. and the fast-growing phosphate-solubilizing bacterium *Bacillus licheniformis*. N_2-fixation and phosphate solubilization both increased in the mixed culture compared to monocultures. Light microscopy revealed that when the two bacterial species were grown on solid medium, they formed one morphotype colony containing both species, whereas in liquid medium, they grew separately. Though enhanced bacterial proliferation was not observed in mixed cultures growing in seawater; the population of both bacterial species increased at the same rate as in monocultures.

Inoculation of black mangrove propagules in artificial seawater with mixed bacterial cultures showed some advantage over inoculation with monocultures; more leaves developed and higher levels of ^{15}N were incorporated in the leaves, however, the total nitrogen level decreased. This illustrates that interactions between microorganisms in the rhizosphere of mangroves can influence plant development and should be considered when applying plant growth-promoting bacteria. These initial works are part of a long-term study designed to assess the feasibility of using terrestrial and marine plant growth-promoting bacteria for the inoculation of marine plants for environmental purposes.

Desert reforestation. When naturally vegetated deserts are cleared to produce marginal agricultural land that is later abandoned (a process often called "desertification"), or to build urban neighborhoods lacking paved roads, nothing remains to prevent the topsoil from being eroded by wind. The result is severe soil erosion and subsequent dust pollution. The latter indirectly but significantly increases chronic respiratory illnesses. This phenomenon has been increasing throughout the developing world. In North America, it is predominant in the semiarid areas of northern Mexico. Abandoned fields in northwestern Mexico quickly become a barren landscape with few, if any, annual plants. These areas cannot reforest naturally with their natural cacti because nurse trees (which provide a canopy for cacti) that are essential for the establishment of cactus seedlings have been removed.

Desert plants, especially cacti, are excellent topsoil stabilizers. These plants could be used to prevent soil erosion and reduce dust in urban areas, however they have low establishment rates and are slow to develop after being transferred from natural habitats or from a nursery to eroded urban soil. Cacti may benefit from inoculation with beneficial microorganisms during planting and immediately thereafter, commons practice in temperate reforestation. Beneficial microorganisms like PGPBs and mycorrhizal fungi may aid the desert revegetation process and help to reduce soil erosion and dust pollution.

Vesicular-arbuscular mycorrhizal (VAM) fungi may help stabilize “resource islands” (soil mounds produced from captured dust) under mesquite trees by promoting the establishment of cacti in the understory. The VAM fungi associated with perennial plants in disturbed (man-made removal of the natural vegetation) and undisturbed large fields in the Sonoran desert near La Paz, Baja California Sur, Mexico were studied. All 46 species of perennial plants tested had VAM associations but the extent of root colonization by the mycorrhizal fungi varied widely (<10 to > 70%). Cactus species with low VAM colonization thrived mainly near nurse trees. Of the nine species of trees and arborescent shrubs in the area, the mature (>20 year) nurse legumes *Prosopis articulata* (mesquite) and *Olneya tesota* (ironwood) supported the largest number of understory plants. The VAM inoculum potential under the mesquite canopy and in areas devoid of plants was similar (1), however the propagule density of VAM under the canopy was 7-fold higher. These studies show that VAM fungi help to stabilize windborne soil that settles under dense plant canopies by formation of soil aggregation and enhance colonization by cactus seedlings (6).

Bacteria may also contribute to the revegetation of disturbed desert areas. Seedlings of the giant cardon cactus (*Pachycereus pringlei*) were inoculated with *A. brasilense* in pot cultures containing different soils ranging from rich soil from under the mesquite canopy to poor soil from barren areas. In rich soil, *A. brasilense* had no effect on cardon cactus development. However, in poor soil, inoculation increased dry vegetative mass by 60% and root length by over 100%. The effect was not caused by N_2 fixation by the bacterium because acetylene reduction activity was not detected in the roots (5).

Survival and development of cactus transplants in urban, disturbed areas of the desert near La Paz, Baja California Sur, Mexico was monitored. Young plants of three species of tree-shaped cacti (*Pachycereus pringlei, Stenocereus thurberi,* and *Lophocereus schottii*) were inoculated with the plant growth-promoting bacterium *A. brasilense* in an eroded area (a dirt road). Inoculated plants had a higher survival rate and developed more rapidly than uninoculated control plants during a 3.5-year period after transplantation. Soil erosion in the inoculated experimental area diminished. Small, but significant, soil accumulation was associated with the growth of cactus small roots in the wind-deposited dust. The upward growth of small roots into the deposited dust during the rainy season was one mechanism for stabilizing the dust. *A. brasilense* survived well in the rhizospheres of these cacti for two years, but not in root-free soil (4).

These studies demonstrate that (i) the natural revegetation process in the desert can be mimicked by revegetation programs, and (ii) the inoculation of cacti with fungi or bacteria can enhance their establishment in disturbed areas, and can thereby stabilize soil.

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References

1. Bashan, Y., Davis, E. A., Carrillo, A., and Linderman, R.G. 2000. Assessment of VA mycorrhizal noculum potential in relation to the establishment of cactus seedlings under mesquite nurse-trees in the Sonoran desert. Appl. Soil Ecol. 14:165-176.
2. Bashan, Y., Moreno, M., and Troyo, E. 2000. Growth promotion of the seawater-irrigated oilseed halophyte *Salicornia bigelovii* inoculated with mangrove rhizosphere bacteria and halotolerant *Azospirillum* spp. Biol. Fertil. Soils 31: (in press)
3. Bashan, Y., Puente, M.E., Myrold, D.D., and Toledo, G. 1998. In vitro transfer of fixed nitrogen from diazotrophic filamentous cyanobacteria to black mangrove seedlings. FEMS Microbiol. Ecol. 26:165-170
4. Bashan, Y., Rojas, A., and Puente, M.E. 1999. Improved establishment and development of three cacti species inoculated with *Azospirillum brasilense* transplanted into disturbed urban desert soil. Can. J. . Microbiol. 45: 441-451
5. Carrillo-Garcia, A., Bashan, Y., Diaz-Rivera, E., and Bethlenfalvay, G.J. 2000. Effects of resource - island soils, competition, and inoculation with *Azospirillum* on survival and growth of *Pachycereus pringlei*, the giant cactus of the Sonoran Desert. Restor. Ecol. 8: 65-73
6. Carrillo-Garcia, A., Leon de la Luz, J.-L., Bashan, Y., and Bethlenfalvay, G.J. 1999. Nurse plants, mycorrhizae, and plant establishment in a disturbed area of the Sonoran desert. Restor. Ecol. 7**:** 321-335
7. Gonzalez, L.E., and Bashan, Y. 2000. Increased growth of the microalga *Chlorella vulgaris* when coimmobilized and cocultured in alginate beads with the plant growth-promoting bacterium *Azospirillum brasilense*. Appl. Environ. Microbiol. 66: 1527-1531
8. Gonzalez-Bashan, L.E., Lebsky, V.K., Hernandez, J.P., Bustillos, J.J., and Bashan, Y. 2000. The metabolism of the microalga *Chlorella vulgaris* is affected by its natural associative bacterium *Phyllobacterium myrsinacearum* when coimmobilized in alginate beads. Can. J. Microbiol. 46: (in press)
9. Puente, M.E., Holguin, G., Glick, B.R., and Bashan, Y. 1999. Root surface colonization of black mangrove seedlings by *Azospirillum halofraeferens* and *Azospirillum brasilense* in seawater. FEMS Microbiol. Ecol. 29: 283-292