

Effect of Inoculation with *Azospirillum brasilense* Strains on the Germination and Seedlings Growth of the Giant Columnar Cardon Cactus (*Pachycereus pringlei*)

MARIA-ESTHER PUENTE and YOAV BASHAN*

*Department of Microbiology, Division of Experimental Biology
The Center for Biological Research (CIB), La Paz, P.O. Box 128, B.C. S.,
Mexico 23000*

Tel. 0052 (682) 53633, Fax 0052 (682) 53625

Received October 22, 1991; Accepted August 11, 1992

Abstract

Wild Cardon cactus seeds were inoculated with *Azospirillum brasilense* strains Cd and Sp-245 to improve seed germination and seedling growth parameters. Differential germination responses were related to the bacterial strain. *A. brasilense* Cd (the type strain for the species *A. brasilense*) significantly decreased seed germination. *A. brasilense* Sp245 (a known beneficial bacteria for cereal plants) significantly increased the germination of the seedlings at the same inoculum concentration. Both bacterial strains improved several plant growth parameters related to seedling survivability (height, diameter, volume and the volume/surface ratio) and decreased the aging process of the seedling spines. Both strains of *A. brasilense* survived in the cactus rhizosphere for about 300 days after seed inoculation. This study adds further evidence that *A. brasilense* should be considered as a non-specific beneficial bacteria and we propose the inoculation of Cardon cactus seeds as a possible means of enhancing seedling growth in nurseries.

Keywords: *Azospirillum*, bacterial inoculation, beneficial bacteria, cactus, Cardon, *Pachycereus*, rhizosphere bacteria, plant-growth-promoting-rhizobacteria

* The author to whom correspondence should be sent

1. Introduction

The endemic giant columnar Cardon cactus (*Pachycereus pringlei*) is one of the most massive cacti of the world and the most distinctive plant and landmark in the Baja California desert of Mexico (Fig. 1). This tree shaped

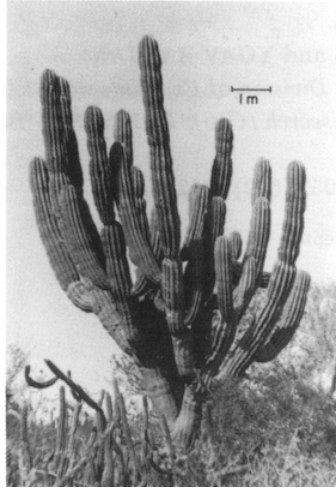


Figure 1. Mature Cardon cactus in the wilderness of the state of Baja California Sur, Mexico. Note the dominance of the plant over the local flora.

cactus normally grows to 15 m, with some specimen reaching over 20 m. The Cardon has a massive trunk over a meter in diameter with up to 70 ascending branches and can weigh more than 25000 kg (Cullmann et al., 1986; Gibson and Nobel, 1986). Although long-lived, each cactus plant annually produces an abundance of viable seeds. The seeds are larger than those of most other columnar cacti (3-4 mm long and 1-2 mm thick); they are edible, highly nutritious (Valencia et al., 1985) and were important in the traditional Seri Indian diet of the Sonoran desert in northern Mexico (Felger and Moser, 1974). However, seedlings establishment under natural conditions is apparently poor; relatively few young plants are detected in southern Baja California peninsula wilderness following their first, hot, dry season. Rare seedling locations can be found in shaded, hard-to-reach crevices, concealed from the ravage of desert rodents. After the initial year, the few surviving young plants are protected against further predation by the production of various toxic compounds and by the hardening of their spines (Franco and Nobel, 1989; Gibson and Nobel,

1986; Valiente-Banuet et al., 1991). This cactus, once established, acts as an excellent top-soil stabilizer with its huge superficial root system preventing dust storms in southern Baja California (Bashan, unpublished data).

The Cardon cactus, known to the western world for hundreds of years and systematically characterized in the beginning of this century (Cullmann et al., 1986), has inspired very little research on its physiology and biochemistry (Mata and McLaughlin, 1986; Tuttle, 1991). Furthermore, its interaction with any soil microorganisms is scarcely documented (Rose, 1981).

The aims of the present study were: (1) to evaluate the effect of two common, cereal-originated *Azospirillum brasilense* strains on the germination and seedling growth of the Cardon cactus, (2) to further establish the notion that *A. brasilense* is a general, non-specific beneficial bacteria, and (3) to present the first report about the effect of *Azospirillum* on a member of the cactus family.

2. Materials and Methods

Organisms

Azospirillum brasilense strains Cd (ATCC 29710) and Sp-245 (Baldani et al., 1986) were used as inoculum. Cardon (*Pachycereus pringlei* (S. Watson) Britton & Rose 1909) seeds were collected from wild cacti located in the natural reserve of the Center for Biological Research (CIB) ("El Comitán" area, 17 km west of La Paz) in August of 1990 and 1991 and kept in hermetically sealed boxes at ambient temperature. As the germination rate of Cardon seeds decreases after a year of storage, each experiment was conducted with the newest seeds available.

Bacterial growth conditions and plant inoculation

Bacteria were grown, maintained, and harvested for inoculation as previously described in detail (Bashan and Levanony, 1985). In general, the bacterial concentration for the inoculum was 10^6 cfu ml⁻¹, an optimal concentration for many plant species (Bashan, 1986b; Bashan et al., 1989a). In a few experiments, other bacterial concentrations (10^4 - 10^7 cfu ml⁻¹) were used. Seeds were inoculated by dipping them for 5 min into bacterial suspensions under a vacuum of 600 mm Hg. Then, the vacuum was released abruptly, allowing the bacteria to penetrate seed cavities which were previously filled with air. Germination experiments were done by either one of the following procedures:

1. Seeds were evenly spread in Petri-dishes (10-50 seeds per 17 cm dish) containing sterile, pre-wetted cotton. The dishes were incubated under constant fluorescent illumination of $70 \mu\text{E m}^{-2} \text{s}^{-1}$ at $25 \pm 3^\circ\text{C}$. (Alcorn and Kurtz (1959) demonstrated the effect of this light and temperature on seed germination of the Saguaro cactus which belongs to the same tribe as the Cardon cactus [Bergman and Bouman, 1983]). Germination was scored daily for 15 days after inoculation.
2. A modification of the "paper towel" method described by Fages and Arsac (1991) was used: seeds were placed on a single, wet paper towel, spread on the bottom of a glass container.

Seedlings growth conditions

Fifteen days after inoculation, the germinating seedlings were aseptically transferred into closed, transparent, plastic trays (25 x 15 x 8 cm) containing sterile, wet, fine vermiculite (6 cm deep). The miniature seedlings were planted 3 cm apart. Each tray consisted of miniature seedlings of one inoculation treatment. Since the seedlings require minimum ventilation, the trays were opened separately, once a week in a laminar flow hood. The nurseries were fertilized with 1/4 Hoagland's nutrient solution every month and incubated up to 11 months under the conditions described above. Measurements of seedling development were taken in intervals ranging from 60 to 270 days after inoculation.

Counting and identifying A. brasilense strains from the rhizosphere

Bacteria were counted from the rhizosphere (the vermiculite fraction which was in close proximity to the roots) or from the paper-grown seedlings as described elsewhere (Bashan et al., 1989a; Krieg and Döbereiner, 1986).

Evaluation of spine age

The qualitative evaluation of spine age was done according to Gibson and Nobel (1986), where white spines are considered juveniles and brown spines are considered mature, due to the production of thick cell-walls which are impregnated with lignin. The respective colors were indexed as follows: 0=presence of only white spines on the seedling; 1=presence of a mixture of white and brown spines on the seedling; 2=presence of brown spines only. The indices presented in Fig. 5F are the means for all the seedlings.

Experimental design, statistical analysis and data presentation

All experiments were performed in a randomized design in 5 replicates. Each replicate consisted of 10-50 seeds. Experiments were repeated up to 12 times each in two consecutive years using a new seed batch every year. Results are means of all experiments. Significance is given by $P < 0.05$. Since wild seeds and seedlings are inherently highly variable, a complete replicate of numerical data is difficult. Only trends can be compared. Therefore, some data are pre-sented as percentage of control seeds and seedlings, i.e., the treatment results were compared to their controls for percentages (in each separate experiment) to be used in statistical analysis after transformation of the data to arcsin.

3. Results*Enhancement and inhibition of germination by the two *A. brasilense* strains at optimal and at various inoculation levels*

Inoculation of Cardon seeds with *A. brasilense* strains Cd and Sp-245 resulted in different responses. In 12 experiments at optimal concentration (10^6 cfu ml⁻¹), strain Cd significantly decreased germination while strain Sp-245 significantly enhanced seed germination (Fig. 2A). Strain Cd significantly inhibited seed germination at concentrations ranging from 10^5 to 10^7 cfu ml⁻¹ (Fig. 2B) while strain Sp-245 significantly enhanced seed germination at concentrations ranging from 10^4 to 10^6 cfu ml⁻¹ (Fig. 2C). Both strains multiplied on the emerging roots, though to a different extent. The Cd strain population increased nearly 10 fold 3 days after inoculation but later decreased. On the other hand, strain Sp-245 continued to multiply up to 8 days after inoculation, reaching very high levels ($> 10^8$ cfu seedling⁻¹) and also decreasing later (Fig. 2D).

*Survival of *A. brasilense* in Cardon rhizosphere*

Both *A. brasilense* strains survived well in the rhizosphere of Cardon seedlings. Although the; bacterial numbers in each replicate varied greatly between the six experiments (ranging from 5×10^4 to 3×10^8 cfu/g vermiculite), all rhizosphere samples contained one of the *A. brasilense* strains one month after inoculation. No cross contamination between inoculated and non-inoculated nurseries was detected (Fig. 3). Further analysis of *A. brasilense* populations revealed a decrease in the size of the population which stabilized more than 2 months after inoculation at a level of 10^3 - 10^4 cfu g vermiculite⁻¹. This level of bacteria for both strains was maintained for at least 300 days (Fig. 3). Since it was assumed that part of the bacterial population remained attached

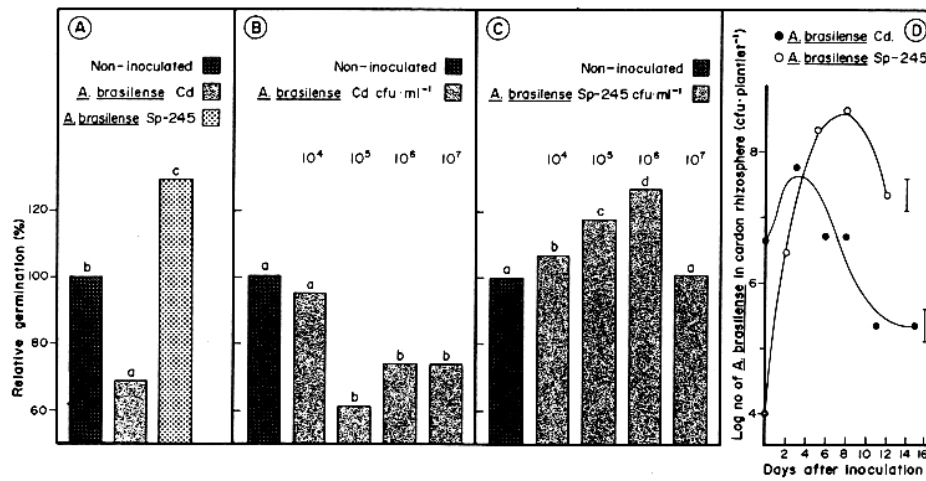


Figure 2. Effect of inoculation on Cardon seeds with *A. brasilense* strains at inoculum level of 10^6 cfu ml⁻¹ (A) and at various inoculum levels (B, C). Results are presented as percentage of a non-inoculated control and are means of 4 (Strain Cd) and 12 (strain Sp-245) experiments (A), 2 experiments (B) and 6 experiments (C), each designed in 5 replicates. Columns followed by a different letter (in each sub-figure, separately) differ significantly at $P = 0.05$; (D) number of bacteria on germinating Cardon seedlings. Bars represent SE of the lines.

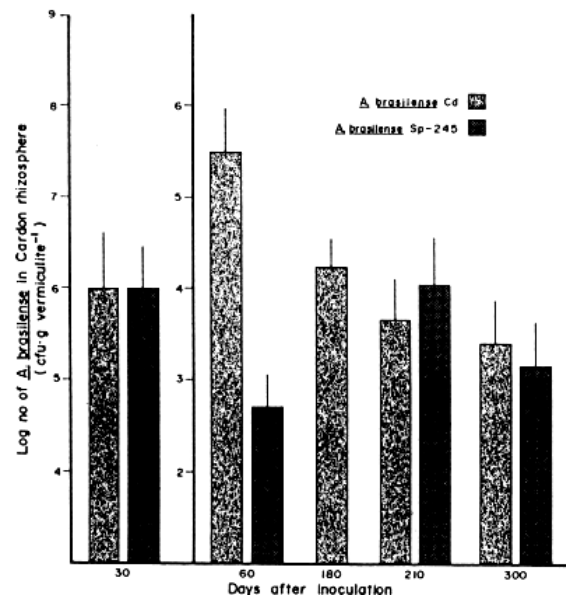


Figure 3. Survival of *A. brasilense*, strains Cd and Sp-245 in Cardon seedling rhizosphere at 30 to 300 days after seed inoculation. Numbers are means of 3-6 replicates. Bars represent SE.

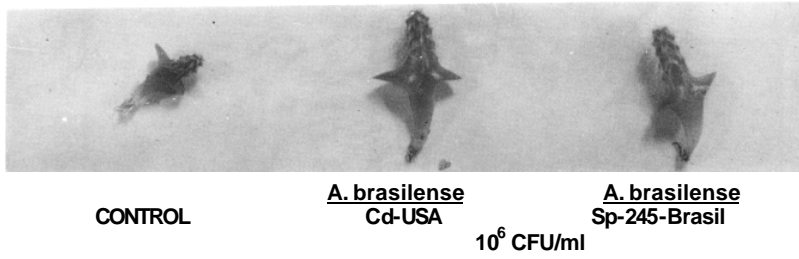


Figure 4. The effect of inoculation of Cardon seeds with 10^6 cfu ml⁻¹ *A. brasilense* strains Cd and Sp -245 on seedling development 5 months after inoculation.

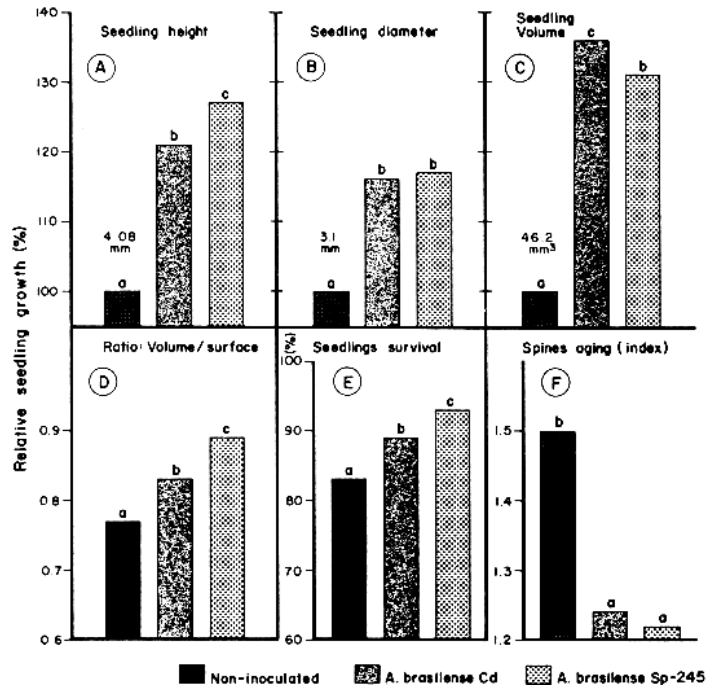


Figure 5. The effect of inoculation with *A. brasilense* on some growth parameters of Cardon seedlings. (A) seedling height; (B) seedling diameter at the center; (C) seedling volume (calculated); (D) ratio volume/surface (calculated); (E) seedling establishment; (F) aging of spines. Numbers which follow columns of non-inoculated plants in sub-figures AC indicate the average size of the seedling in mm (A-B) and mm³ (C). Columns followed by a different letter at each sub-figure differ significantly at P = 0.05 in ANOVA one-way analysis.

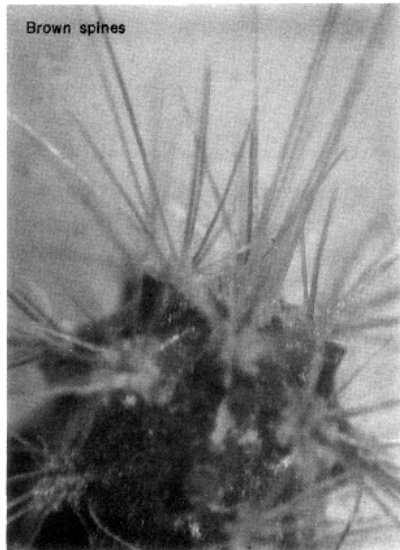


Figure 6a. (A) Non-inoculated. Cardon spines. Brown spines = mature stage of growth.



Figure 6b. Aging of Cardon spines after inoculation with *A. brasilense* Cd. White spines = juvenile stage.

to the vermiculite particles and were thus uncounted, these numbers should be regarded as minimal counts.

Enhanced growth of inoculated Cardon seedlings

Cardon seedlings significantly and positively responded to *A. brasilense* inoculation (Fig. 4). Both strains enhanced growth parameters such as seedling height (Fig. 5A), diameter (Fig. 5B), volume (Fig. 5C) and the ratio of volume/surface (Fig. 5D). Under nursery conditions, seedling establishment was very high, both in inoculated and non-inoculated nurseries (Fig. 5E).

Inhibition of the aging process of spines

Inoculation of the seeds with either strain resulted in the inhibition of the aging process of the spines. A large portion of the plants still exhibited white spines up to 9 months after inoculation (Fig. 6A), while spines in noninoculated plants became light brown as they normally do in this cactus species (Figs. 5F, 6B).

4. Discussion

Azospirillum strains have been routinely isolated from various plant rhizospheres (Bashan and Levanony, 1990) including cacti (Mascarua-Esparza et al., 1988). This genus is notorious for its beneficial effects on cultivated cereal plants (Bashan, 1986a; Jagnow, 1987) vegetables, and commercial plants (Bashan and Levanony, 1990; Bashan et al., 1989a; Del Gallo and Fabbri, 1990; Fages and Arsac, 1991), and has been known to produce an association with several succulent desert plants (Rao and Venkateswarlu, 1982). This study provides the first evidence that cereal-originated *A. brasilense* strains can positively affect cactus plants as well, and it is one of the few studies demonstrating the effect of *Azospirillum* on wild plants.

Azospirillum strains have been claimed to possess some plant specificity towards cereals (Baldani and Döbereiner, 1980; Kapulnik et al., 1981; Pereira et al., 1988). This study, together with several others (Bashan, 1991; Bashan et al., 1989a,b, 1990, 1992; Del Gallo and Fabbri, 1990; Fages and Arsac, 1991; Hadas and Okon, 1987; Saha et al., 1985) demonstrate the beneficial effects of *Azospirillum* on plants other than the originally intended cereals. It strongly supports the notion that *Azospirillum* should be considered a non-specific, general, beneficial bacteria. As such, strains of this genus should be tested for inoculating a large variety of commercial plant families and should not be limited to cereals where their effect is inconsistent and unpredictable (Bashan and Levanony, 1990; Jagnow, 1987).

The *Azospirillum* mode of action is far from being understood and various proposals are pending, including one that claims there is no major mechanism. Instead, the effect is attributed to several, small magnitude mechanisms which participate in the association, the sum of which results in the observed changes in plant growth (Bashan and Levanony, 1990). The results presented in this study do not provide new insight into this controversy. Conversely, by presenting extensive data collected from many repetitive experiments, it further emphasizes that the complexity of *Azospirillum*-plant interaction is not limited to cereals alone, but to other plant species as well. It is as yet unclear, for example, how the bacteria produce different responses on the germination process (inhibition vs. enhancement by different strains) and yet, both strains enhance seedling growth. Apparently, the theory that high levels of bacteria produce "toxic effects" on plants (Barbieri et al., 1988; Bashan, 1986b; Kapulnik et al., 1985) does not hold for all *A. brasilense* strains; *A. brasilense* Sp-245 is capable of developing extremely large populations on Cardon roots, yet still enhances seedling growth.

Full-grown Cardon cacti are highly adaptable to the harsh arid zones of

Baja California. However, to survive the first hot, dry season, the miniature seedling must develop sufficient water storage tissue during the preceding wet season. A spherical shape is the ultimate form for water conservation since it maximizes the volume of stored water per unit area of transpiring surface. Thus, a higher ratio of volume/surface is of utmost importance and the key parameter for seedling survival; consequently, a larger seedling can successfully endure a longer drought (Jordan and Nobel, 1981; Gibson and Nobel, 1986). Inoculation with *A. brasilense*, which increased plant growth parameters related to seedling survival, is important to future nurseries of Cardon destined for the re-forestation of desertified regions. These results may also provide clues for improving seedling establishment which is at present, extremely low.

The observed decrease in the aging process of spines is an apparent disadvantage to the survival of the young seedling. These spines are not producing thick cell-walls and accumulate less lignin, thus remaining alive arid bristle. Lignified, hard spines, provide the first line of defense for the seedling against consumption by desert rodents. Seedling predation, even under normal growth conditions, destroys more than 99% of the seedlings in their first year (Gibson and Nobel, 1986). Therefore, the phenomenon of prolonged spine juvenation certainly requires further attention.

In conclusion, the inoculation of Cardon seeds with *A. brasilense* strains affects seed germination and promotes seedling development.

Acknowledgements

This study was written for the memory of the late Mr. Avner Bashan from Israel. We thank Dr. J. Döbereiner, EMBRAPA, Brazil for donating *A. brasilense* Sp-245, Dr. Fernando Garcia Carreño for launching the idea of cactus inoculation; Mr. Roy Bowers for careful English corrections and Mr. Oscar Armendariz-Ruiz for art work. This study was partially supported by the Consejo Nacional de Ciencia y Tecnología (CONASCYT), Mexico, grant #D000/1178. '

REFERENCES

- Alcorn, S.M. and Kurtz, E.B., Jr. 1959. Some factors affecting the germination of seed of the saguaro cactus (*Carnegiea gigantea*). *Am. J. Bot.* **48**: 526-529.
- Baldani, V.L.D., Alvarez, M.A. de B., Baldani, J.I. and Döbereiner, J. 1986. Establishment of inoculated *Azospirillum* spp. in the rhizosphere and in roots of field grown wheat and sorghum. *Plant Soil* **90**: 35-46.
- Baldani, V.L.D. and Döbereiner, J. 1980. Host-plant specificity in the infection of cereals with *Azospirillum* spp. *Soil Biol. Biochem.* **12**: 433-439.

- Barbieri, P., Bernardi, A., Galli, E., and Zanetti, G. 1988. Effects of inoculation with different strains of *Azospirillum brasilense* on wheat roots development. In: *Azospirillum IV, Genetics, Physiology, Ecology*. W. Klingmüller, ed. Springer Verlag, Berlin, pp. 181-188.
- Bashan, Y. and Levanony, H. 1985. An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense*. *Can. J. Microbiol.* **31**: 947-952.
- Bashan, Y. 1986a. Enhancement of wheat roots colonization and plant development by *Azospirillum brasilense* Cd. following temporary depression of the rhizosphere microflora. *Appl. Environ. Microbiol.* **51**: 1067-1071.
- Bashan, Y. 1986b. Significance of timing and level of inoculation with rhizosphere bacteria on wheat plants. *Soil Biol. Biochem.* **18**: 297-301.
- Bashan, Y. and Levanony, H. 1988. Active attachment of *Azospirillum brasilense* Cd to quartz sand and to a light-textured soil by protein bridging. *J. Gen. Microbiol.* **134**: 2269-2279.
- Bashan, Y., Ream, Y., Levanony, H., and Sade, A. 1989a. Nonspecific responses in plant growth, yield, and root colonization of noncereal crop plants to inoculation with *Azospirillum brasilense* Cd. *Can. J. Bot.* **67**: 1317-1324.
- Bashan, Y., Singh, M., and Levanony, H. 1989b. Contribution of *Azospirillum brasilense* Cd to growth of tomato seedlings is not through nitrogen fixation. *Can. J. Bot.* **67**: 2429-2434.
- Bashan, Y. and Levanony, H. 1990. Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture. *Can. J. Microbiol.* **36**: 591-608.
- Bashan, Y., Harrison, S.K., and Whitmoyer, R.E. 1990. Enhanced growth of wheat and soybean plants inoculated with *Azospirillum brasilense* is not necessarily due to general enhancement of mineral uptake. *Appl. Environ. Microbiol.* **56**: 769-775.
- Bashan, Y. 1991. Changes in membrane potential of intact soybean root elongation zone cells induced by *Azospirillum brasilense*. *Can. J. Microbiol.* **37**: 958-963.
- Bashan, Y., Alcaraz-Melendez, L., and Toledo, G. 1992. Responses of soybean and cowpea root membranes to inoculation with *Azospirillum brasilense*. *Symbiosis* **13**: 217-228.
- Bergman, R. and Bouman, F. 1983. Seed germination in Cactaceae. *Bot. J. Linnean Soc.* **86**: 357-374. .
- Cullmann, W., Götz, E., and Gröner, G. 1986. *The Encyclopedia of Cacti*. Alphabooks, Sherborne, Dorset, England, p. 266.
- Del Gallo, M. and Fabbri, P. 1990. Inoculation of *Azospirillum brasilense* Cd on chick pea (*Cicer arietinum*). *Symbiosis* **9**: 283-287.
- Fages, J. and Arsac, J.F. 1991. Sunflower inoculation with *Azospirillum* and other plant growth promoting rhizobacteria. *Plant Soil* **137**: 87-90.
- Felger, R.S. and Moser, M.B. 1974. The traditional pharmacopoeia of the Seri Indians of Sonora, Mexico. *Econ. Botany* **28**: 414-436.
- Franco, A.C. and Nobel, P.S. 1989. Effect of nurse plants on the microhabitat and growth of cacti. *J. Ecol.* **77**: 870-886.

- Gibson, A.C. and Nobel, P.S. 1986. *The Cactus Primer*. Harvard University Press, Cambridge, MA, p. 286.
- Hadas, R. and Okon, Y. 1987. Effect of *Azospirillum brasilense* inoculation on root morphology and respiration in tomato seedlings. *Biol. Fertil. Soils* **5**: 241-247.
- Jagnow, G. 1987. Inoculation of cereal crops and forage grasses with nitrogen-fixing rhizosphere bacteria: a possible cause of success and failure with regard to yield response - a review. *Z. Pflanzenernaehr. Dueng. Bodenkde.* **150**: 361-368.
- Jordan, P.W. and Nobel, P.S. 1981. Seedling establishment of *Ferocactus acanthodes* in relation to drought: Ecology **62**: 901-906.
- Kapulnik, Y., Sarig, S., Nur, L., Okon, Y., Kigel, J., and Henis, Y. 1981. Yield responses in summer cereal crops of Israeli fields inoculated with *Azospirillum*. *Exp. Agric.* **17**: 179-187.
- Kupulnik, Y., Okon, Y., and Henis, Y. 1985. Changes in root morphology of wheat caused by *Azospirillum brasilense*. *Can. J. Microbiol.* **31**: 881-887.
- Krieg, N.R. and Döbereiner, J. 1986. The genus *Azospirillum*. In: *Bergey's Manual of Systematic Bacteriology*. N.R. Krieg and J.G. Holt, eds. Vol. 1. Williams and Wilkins Co., Baltimore, MD, pp. 96-104.
- Mascarua-Esparza, M.A., Villa-Gonzalez, R., and Caballero-Mellado, J. 1988. Acetylene reduction and indoleacetic acid production by *Azospirillum* isolates from Cactaceous plants. *Plant Soil* **108**: 91-95.
- Mata, R. and McLaughlin, J.L. 1980. Tetrahydroisoquinoline alkaloids of the Mexican columnar cactus *Pachycereus weberi*. *Phytochemistry* **19**: 673-678.
- Pereira, J.A.R., Cavalcante, V.A., Baldani, J.L., and Döbereiner, J. 1988. Field inoculation of sorghum and rice with *Azospirillum* spp. and *Herbaspirillum seropedicae*. *Plant Soil* **110**: 269-274.
- Rao, A.V. and Venkateswarlu, B. 1982. Associative symbiosis of *Azospirillum lipoferum* with dicotyledonous succulent plants of the Indian desert. *Can. J. Microbiol.* **28**: 778-782.
- Rose, S.L. 1981. Vesicular- arbuscular endomycorrhizal associations of some desert plants of Baja California. *Can. J. Bot.* **59**: 1056-1060.
- Saha, K.C., Sannigrahi, S., and Mandal, L.N. 1985. Effect of inoculation of *Azospirillum lipoferum* on nitrogen fixation in rhizosphere soil, their association with roots, yield and nitrogen uptake by mustard (*Brassica juncea*). *Plant Soil* **87**: 273-280.
- Tuttle, M.D. 1991. Bats- the cactus connection. *National Geograph.* **179**: 130-140.
- Valencia, M.E., Atondo, J.L., and Hernandez, G. 1985. Nutritive value of *Zostera marina* and Cardon (*Pachycereus pringlei*) as consumed by the Seri Indians in Sonora Mexico. *Ecol. Food Nutr.* **17**: 165-174.
- Valiente-Banuet, A., Vite, F., and Zavala-Hurtado, J.A. 1991. Interaction between the cactus *Neobuxbaumia tetetzo* and the nurse shrub *Mimosa luisana*. *J. Veg. Sci.* **2**: 11-14.