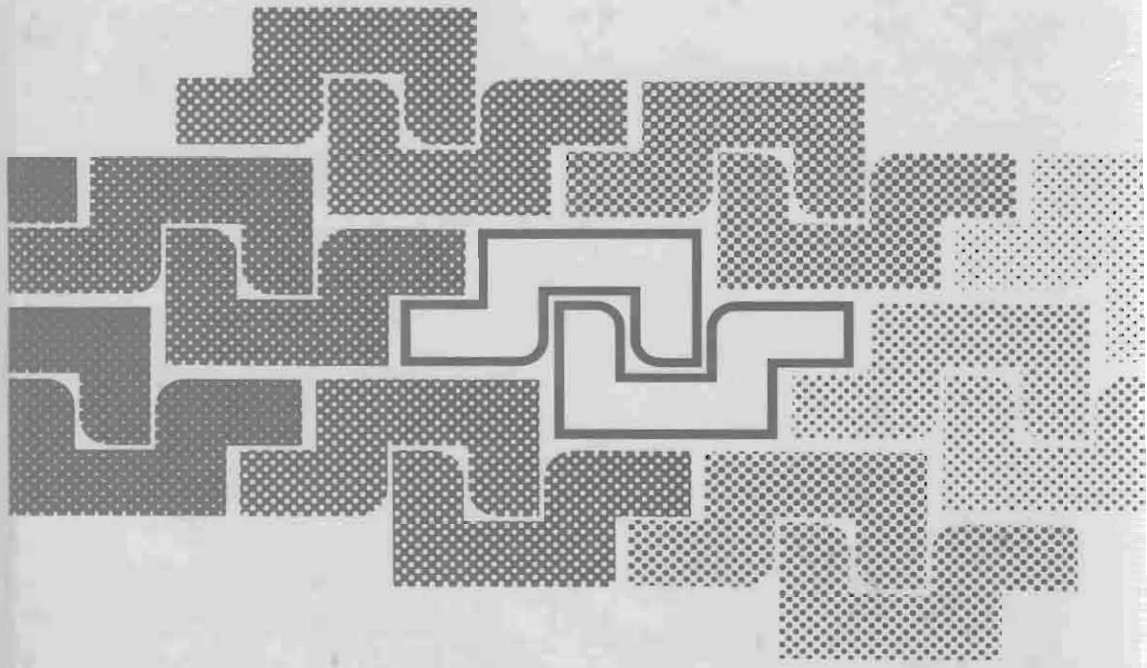

Azospirillum VI and Related Microorganisms

Genetics – Physiology – Ecology

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Soil Parameters which Affect the Survival of *Azospirillum brasilense*

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Abstract

The survival of *Azospirillum brasilense* strains Cd and Sp-245 was evaluated in the rhizosphere of wheat and tomato plants and in 23 plant-free soils of 23 soil types obtained from a wide range of environmental conditions in Israel and Mexico. The survival rate of *A. brasilense* was analyzed for 15 soil parameters. *A. brasilense* survived well in all the rhizospheres tested, regardless of soil type, bacterial strain, the origin of the soil, or the amount of rainfall each soil received prior to sampling. In the absence of plants, the general survival characteristics of *A. brasilense* differed and were related mainly to the geographical origin of the soil and not to prevailing environmental conditions. We propose that: (i) *A. brasilense* is a rhizosphere colonizer which survives poorly in most soils for prolonged periods. (ii) Some major physical and chemical soil parameters may affect survival of the bacteria in plantless soils.

Introduction

There is no doubt that *Azospirillum* species survive well and for prolonged periods of time in the rhizosphere of numerous plant species (Bashan and Levanony, 1990). However, survival in the soil *per se* is controversial. On one hand, several studies, mainly (Baldani et al. 1987; Döbereiner and Baldani, 1979; Döbereiner et al. 1976; Sadasivam et al. 1986; Shawky, 1989) but not exclusively (Germida, 1986) from tropical areas, have indicated that *Azospirillum* was found in nearly every sampling of soil, indicating a high capacity for survival. On the other hand, studies mainly from temperate and semi-arid zones, but also from tropical regions (Nayak et al. 1986) found that *Azospirillum* survived poorly in these soils (Albrecht et al. 1983; Bashan, 1986 a; Bashan et al. 1987; De Coninck et al. 1988; Smith et al. 1984; Vandenhove et al. 1993) and hardly lasted from one season to the next (Harris et al. 1989).

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Overwintering of inoculated *Azospirillum* is a crucial negative factor for both the inoculation industry (one inoculation should not last for more than one season) and the growers (competition with the previous year's, inferior strains).

The main controversy originated from the diverse soil types used in the studies. No two studies used the same soil. Furthermore, these soil types were not compared with those of other studies and even with those of the same region. This controversy in the soil survival of *Azospirillum* was recognized as one of the basic unsolved questions of *Azospirillum* research (W. Klingmüller, General discussion, *Azospirillum* V workshop, 1991, Germany).

The aim of this study was to: challenge the controversy by correlating the soil parameters of 23 soil types from 4 distinct regions (arid, semi-arid and mountain soils from Israel, semi-arid and tropical soils from Central Mexico, and arid soils from Baja California, Mexico) with the survival of two common strains of *Azospirillum brasilense*, Cd and Sp-245.

Material and methods

Organisms

For all experiments we used *Azospirillum brasilense* strains Cd (ATCC 29710) and Sp-245 (Baldani et al. 1986) with the plants wheat (*Triticum aestivum*) cvs. "Deganit" (Israel) and "Morelos" (Central Mexico) and tomato (*Lycopersicon esculentum*) cv. "UC-82-L" (Baja California Sur, Mexico).

Bacterial inoculation

Bacteria were grown in one of the following growth media: Nutrient Broth (in Baja California, Mexico and Israel) or N-free medium (NFb) (in mainland Mexico) and prepared for inoculation at various concentrations as previously described (Bashan, 1986 b, Bashan et al. 1993). The final bacterial concentrations were: $1x10^6$ cfu/ml soil either in the presence or absence of plants in Israel, $1.77x10^7$ cfu/ml soil for both soil or plant inoculation in Baja California, Mexico, and $4.2x10^4$ (to inoculate plants) or $1.44x10^8$ cfu/ml (to inoculate soil) in Central Mexico. Soil was directly inoculated by applying double washed bacterial suspension to each pot. Plants were inoculated at sowing as previously described (Puente and Bashan, 1993).

Soil analyses

Twenty three soil types were collected in various regions of Israel and Mexico and kept in hermetically sealed plastic containers at 4±1°C. All soil samples were collected by commercial core samplers from the soil layer 20-30 cm deep after discarding the top soil. No attempt was made to preserve the soil intact and therefore, all samples should be considered disturbed. Several soils were from cultivated areas and the others were from uncultivated land. Soils were collected from arid (< 200 mm rainfall / year)(11 soils), semi-arid (400-600 mm rainfall /year) (5 soils), mountains (500-800 mm rainfall /year)(6 soils), and tropical (1800+ mm rainfall /year) (one soil).

The physical and chemical characteristics of each soil were determined using soil analysis standard methods: texture and organic matter (Royce, 1980); pH, water holding capacity and electric conductivity (Chapman and Pratt, 1984); nitrogen content (Volonteri, 1983); and $CaCo_3$ content (Jackson, 1976).

Soil sterilization

To avoid competition with native microorganisms and possible complications in the final analysis, all soils were sterilized by a standard tyndelization procedure (1 h at 15 lbs in an autoclave. Later, the soil was incubated for 24 h at 30±1°C. This was repeated 3 times). Preliminary comparison between sterile and non-sterile soil showed no significant difference in the level of survival for both strains Cd and Sp-245.

Plant growth conditions and inoculation

Plants were grown in 500 ml plastic pots containing 400 g of soil. All pots were disinfected with 10% NaOCl and thoroughly washed with sterile tap water before use. Inoculation of soils without plants was carried out in identical 250 ml pots. Plants were grown in a growth chamber at 25±1°C, 100 mole/m^2/sec and 60±2 % relative humidity. Plants were irrigated every week with 5-15 ml sterile, distilled water to avoid saturation as required by the different size of the growing plants. Plants were fertilized once a week with 5 ml, half-strength Hoagland's solution.

Sampling and bacterial counts from soil and root samples

Samples (2 g soil or approx 500-1000 mg (fresh weight) roots and the adhering soil particles) were taken at each sampling. Rhizosphere bacteria were considered as the bacteria that colonize the roots and the adhering soil particles which were not removed. The samples were lightly sonicated at 25 W for 5 min (Cole Parmer Series 4710, USA) and then decimally

diluted in 0.06M Phosphate buffer pH 7.0. Bacteria from the soil were counted after similar treatment by conventional plate count method on Nutrient Agar (soils from Baja California, Mexico) or on N-free NFb medium (Bashan et al. 1993)(soils from Central Mexico), by indirect-ELISA (soils from Israel) (Levanony and Bashan, 1991) or by the time-limited liquid enrichment technique (Bashan et al. 1991) when the number of bacteria fell below the level of detection by the ELISA ($<10^4$ cfu/sample).

Experimental design and statistical analysis

All experiments were carried out in 3-4 replicates per treatment. The rate of growth or death of the bacterial population was calculated according to Krebs' Logistic Equation of Growth (Krebs, 1978).

Primarily, the rate of growth/death of the bacteria in each of the soils were correlated with the values obtained for each soil parameter in each of 23 different type of soils using Linear and Multiple Regression Analyses at $P \leq 0.01$ and 0.05. Then, all the survival data from all the soils and all the soil parameter data were analyzed together by Principle Component Analysis procedure.

Results and discussion

The release of *Azospirillum* and other PGPR into soils has a long history of unpredictable and often disappointing results. One of the main obstacles has been the often poor establishment and survival of the introduced bacteria in the soil prior to root colonization (Bashan and Levanony, 1990, Michiels et al. 1989, Jagnow, 1987). Survival of PGPR in the soil is a crucial issue for both inoculant users and manufacturers, stemming from the common agrotechnical belief that seed inoculation is impractical in many cases (perennial plants, vegetative propagated plants, trees, or where more than one inoculation per season is required, etc). When the PGPR is applied to the soil, it is hoped that it will survive long enough to find its target plant. Thus, before one considers an expensive field inoculation, the prospect of bacterial survival in the soil must be considered.

The aim of this study was to address the soil phase survival of *Azospirillum* by creating sufficient data which will allow future modeling and prediction of bacterial behavior in any given soil without laborious studies of bacterial survival in every field. To this end, we collected 23 soil types representing different climatological conditions, from tropical to arid

zones, and compared them all in identical, commonly used experiments using the most available stains of *A. brasilense*.

One fact emerged immediately and was clear even without the aid of any statistical analysis. *A. brasilense* proved to be a rhizosphere bacteria. It survived well regardless of any of the soil characteristics as long as plants were growing in these soils. In the absence of plants, the survival picture differed significantly. The general survival differences were more geographical than climatological, i.e., in the arid soils of Israel the bacteria poorly survived while it proliferated in the arid soils of Baja California. An example for this tendency is given in Fig. 1 in which the survival of *A. brasilense* in the rhizosphere and soil in three representative soil types is demonstrated.

With the aid of several statistical analyses performed on the bacterial survival data and the soil parameters data, we were able to sort out the major soil factors affecting the survival of *A. brasilense* in these soils. Two factors, the levels of $CaCO_3$ and rough sand were negatively and significantly correlated with bacterial survival (Fig 2).

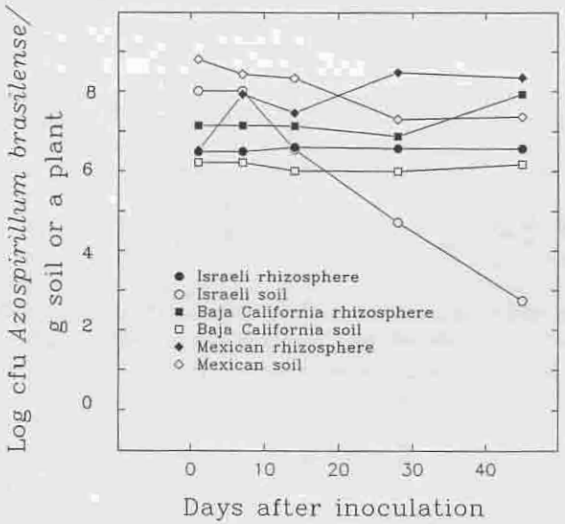

Fig. 1. Survival of *Azospirillum brasilense* in the soil and rhizosphere of 3 soil types obtained from Israel, Baja California Sur, Mexico and Central Mexico.
Each point represents the mean of 3-5 independent samplings from pots, each conducted in triplicates. For simplicity, the standard deviations were not drawn and are: Israeli soil; rhizosphere (R) ±1.4851, soil (S) ±1.5275. Baja California soil; R ±1.39024, S ±1.5149. Central Mexico soil; R ±1.24098, S ±1.5127.

No other single parameter was responsible by itself for survival. However, Principle Component Analysis revealed that when several factors were grouped together (clay, nitrogen, organic matter and water holding capacity), they positively affected survival. On the other hand, levels of $CaCO_3$ and rough sand together with the level of fine sand in the soil had a negative influence on the survival of the bacteria. The effects of single soil parameters on the survival and proliferation of soil and rhizosphere bacteria are known (Foster, 1988; van Elsas, 1992; van Elsas and van Overbeek, 1993). The novelty of this study is that only the combined action of several, lessor parameters significantly and positively determined the survival of *Azospirillum* in the soil. Nevertheless, these parameters are unimportant when plants are growing in the same type of soils. What is still unknown are the proportional effects of each parameter on theoverall effect, and whether manipulation of the soil parameters will alter the bacterial survival.

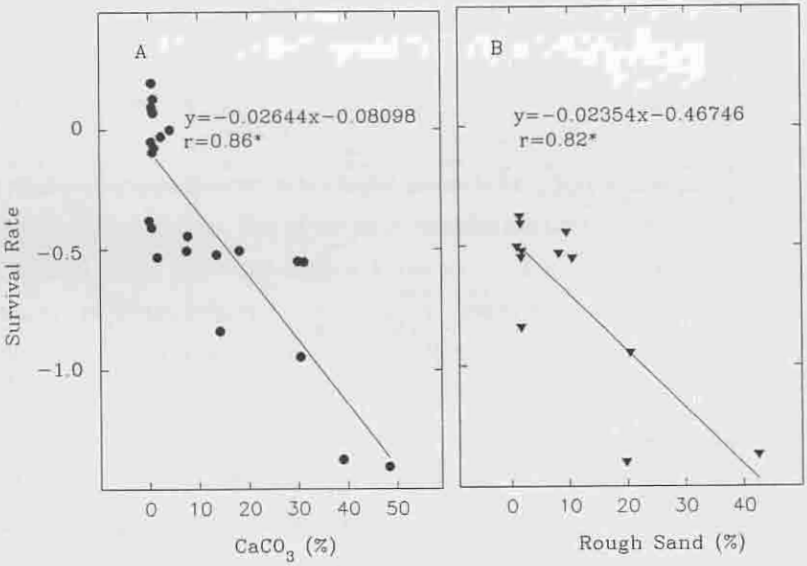

Fig. 2. Linear regression analyses between percentage of $CaCO_3$ (A) and rough sand (B) and the survival rate of *A. brasilense* in 23 and 13 soil types, respectively. Missing points in each sub-figure are overlapped by other printed points. * - Significance of the regression at $P \leq 0.05$.

In conclusion, we propose that: (i) *A. brasilense* is a rhizosphere colonizer which survives poorly in most soils for prolonged periods and, (ii) some major physical and chemical soil parameters may affect the survival of this bacteria in plantless soils.

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