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Survival of *Azospirillum brasilense* in the Bulk Soil and Rhizosphere of 23 Soil Types†

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The survival of *Azospirillum brasilense* Cd and Sp-245 in the rhizosphere of wheat and tomato plants and in 23 types of plant-free sterilized soils obtained from a wide range of environments in Israel and Mexico was evaluated. Large numbers of *A. brasilense* cells were detected in all the rhizospheres tested, regardless of soil type, bacterial strain, the origin of the soil, or the amount of rainfall each soil type received prior to sampling. Survival of *A. brasilense* in soils without plants differed from that in the rhizosphere and was mainly related to the geographical origin of the soil. In Israeli soils from arid, semiarid, or mountain regions, viability of *A. brasilense* rapidly declined or populations completely disappeared below detectable levels within 35 days after inoculation. In contrast, populations in the arid soils of Baja California Sur, Mexico, remained stable or even increased during the 45-day period after inoculation. In soils from Central Mexico, viability slowly decreased with time. In all soils, percentages of clay, nitrogen, organic matter, and water-holding capacity were positively correlated with bacterial viability. High percentages of $CaCO_3$ and fine or rough sand had a highly negative effect on viability. The percentage of silt, pH, the percentage of phosphorus or potassium, electrical conductivity, and C/N ratio had no apparent effect on bacterial viability in the soil. Fifteen days after removal of inoculated plants, the remaining bacterial population in the three soil types tested began to decline sharply, reaching undetectable levels 90 days after inoculation. After plant removal, percolating the soils with water almost eliminated the *A. brasilense* population. Viability of *A. brasilense* in two artificial soils containing the same major soil components as the natural soils from Israel did was almost identical to that in the natural soils. We conclude that *A. brasilense* is a rhizosphere colonizer which survives poorly in most soils for prolonged periods of time; that outside the rhizosphere, seven abiotic parameters control the survival of this bacterium in the soil; and that disturbance of the soil (percolation with water or plant removal) directly and rapidly affects the population levels.

Azospirillum species survive for prolonged periods of time in the rhizosphere of numerous plant species (13). Colonization of roots is nonspecific, and bacteria migrate between different plant species (7, 9, 18). However, conflicting evidence has been reported for survival of *Azospirillum* spp. in the soil outside the rhizosphere. *Azospirillum* spp. occur in most soils of tropical (3, 22, 23, 48, 51) and some soils of temperate (26) regions, indicating a high survivability outside the rhizosphere. In contrast, in studies done mainly in temperate and semiarid zones (1, 4, 15, 21, 52, 53), but also in tropical regions (41), it was found that introduced *Azospirillum* spp. survived poorly in these soils and hardly lasted from one season to the next (27). In Israeli soils, *Azospirillum* spp. adsorbed firmly to soil particles, especially clays and organic matter in the topsoil, but barely washed downward (11, 12). In temperate soils and under conditions of water stress or old bacterial age, the bacterium took on a cyst-like form which is believed to be more resistant than the common vegetative cells and therefore may serve as a survival form (14, 32, 49, 50). In sandy soils, the bacterium produced fibrillar material which immobilized it to a specific microenvironment (16, 34). This particular feature of *Azospirillum brasilense* differentiates it from several other plant-growth-promoting rhizobacterium-like biocontrol pseudomonads, which can wash down with percolating water (36).

The soil specimens were different in all the soil survival studies, which probably accounts for the disparity in results. Furthermore, types and characteristics of soil used in the different studies have never been compared. This was due largely to complications involving the transportation of large volumes of soil over international borders. Survival of *Azospirillum* spp. in soil has been recognized as one of the basic unsolved questions in *Azospirillum* research (30a).

The aims of the present study were to provide insights into bacterial survival by correlating the soil parameters of 23 soil types from distinct regions (arid, semiarid, and mountain soils from Israel; semiarid soils and a tropical soil from mainland central Mexico; and arid soils from Baja California, Mexico) with the survival of two common strains of *Azospirillum brasilense*, Cd and Sp-245, to evaluate the effects of changing environmental conditions (such as plant removal and water percolation through soil) on survival, and to compare the survival rates of *A. brasilense* in natural soils and artificial soils (AS) with the same major abiotic components.

MATERIALS AND METHODS

Organisms. All experiments were done with *A. brasilense* Cd (ATCC 29710) and Sp-245 (2). The plants used were *Triticum aestivum* 'Deganit' (Israel) and 'Morelos' (mainland Mexico) (wheat) and *Lycopersicon esculentum* 'UC-82-L' (Baja California Sur, Mexico) (tomato).

Bacterial inoculation. Bacteria were grown in either nutrient broth (for introduction into soils of Baja California and Israel) or N-free medium (NFb) (for mainland Mexico soils) and prepared for inoculation at various concentrations as

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TABLE 1. Physical properties and chemical compositions of 23 soil types from Israel, Baja California Sur, Mexico, and mainland central Mexico[a]

Soil type	Soil no.	Nitrogen (%)	Phosphorus (%)	Potassium (%)	Clay (%)	Silt (%)	Fine sand (%)	Rough sand (%)	Field capacity (%)	$CaCO_3$ (%)	pH	Conductivity (mS/cm)	Organic matter (%)
Tera Rosa[b] (Rhodoxeralfs) (I)	1	0.18	0.05	1.79	48.9	22.1	27.4	1.6	41.2	0.5	7	0.5	2.76
Mediterranean brown forest[b] (Haploxeralfs) (I)	2	0.24	0.16	0.69	64	12.4	14	9.6	42.3	7.8	7.5	0.9	3.87
Rendzina of mountains[b] (Rendolls) (I)	3	0.13	0.24	0.25	31.3	31.6	35.4	1.7	32.2	30	7.8	0.45	3
Brown basaltic[b] (Xerorthents) (I)	4	0.11	0.21	0.78	40.2	22.3	36	1.5	30	0	6.9	0.37	2.5
Brown red sandy[b] (Haploxeralfs) (I)	5	0.04	0.05	1.25	4.3	5.6	69.6	20.5	8.6	30.5	8.5	0.53	1.3
Brown alluvial-vertisols (I)[b] (Chromoxererts)	6	0.12	0.15	1.3	65.9	18.7	14.4	1	47.5	7.6	8.1	0.85	1.4
Alluvial[b] (Xerofluvents) (I)	7	0.06	ND	3	32.4	7.2	58.6	1.8	29.2	13.5	7.7	0.85	2.2
Brown steppe[b] (Calcixerolls) (I)	8	0.06	ND	2.7	50.6	20.4	28	1	35.7	18.1	8.3	0.67	0.7
Rendzina of valleys[b] (Calciorthids) (I)	9	0.18	0.25	0.83	35.9	29.5	24	10.6	41.4	31.2	7.8	0.87	3.2
Hammada of mountains[b] (Gypsiorthids) (I)	10	0.03	0.85	0.71	20.1	59.3	17.8	19.8	14.4	48.4	7.4	0.71	0.4
Brown desert skeletal[b] (Torriorthents) (I)	11	0.02	0.1	1.08	12.3	21.7	55.5	42.5	16.6	39.1	8	0.9	0.4
Loess raw[b] (Camborthids) (I)	12	0.05	0.12	1.31	14.6	14.8	68.9	1.7	17.1	14.2	8	0.4	0.9
Loessial sandy (I)[b] (Torripsamments)	13	0.02	0.03	0.77	2.3	1	88.4	8.3	3.1	1.6	7.8	0.33	0.3
Eutric Rhegosol with coarse texture (BCS)[b]	14	0.15	0.31	0.13	6.91	17.4	75.6	ND	16.5	0.57	7	0.17	2.14
Eutric Rhegosol with coarse texture (BCS)[c]	15	0.15	2.1	0.19	7.58	26	66.4	ND	17.26	0.57	7.2	0.95	2.82
Eutric Rhegosol with coarse texture (BCS)[b]	16	0	ND	ND	0	0	100	ND	9.8	4.29	7.3	0.77	0
Haplic Yermosol plus Eutric Rhegosol plus Calcaric Rhegosol with medium texture (BCS)[c]	17	0.23	ND	ND	10.32	51.6	38	ND	19.9	1.1	7.7	0.32	2.16
Haplic Yermosol plus Calcaric Rhegosol with coarse texture (BCS)[c]	18	0.8	ND	ND	11.55	38.5	49.9	ND	17.1	0.72	7.4	1.07	1.44
Haplic Xerosol plus Eutric Rhegosol with coarse texture (BCS)[b]	19	0.86	0.79	0.23	11.8	40	48.2	ND	10.4	0.86	7	0.17	3.43
Haplic Xerosol plus Eutric Rhegosol with coarse texture (BCS)[c]	20	0.14	1.68	0.3	10.53	53.3	36.2	ND	13.5	0.86	7.5	0.5	2.49
Haplic Phaeozem plus Pellic Vertisol plus Calcaric Phaeozem (M)[c]	21	0.19	ND	ND	43	36	21	ND	20	0.5	7.4	0.46	2.8
Pellic Vertidol plus Chromo Vertisol plus calcareous Phaeozem with fine texture (M)[c]	22	0.08	ND	ND	29	20	51	ND	34	0.73	6.1	0.28	1.33
Chromo Vertisol plus pellic Vertisol plus Vertic Cambisol with fine texture (M)[c]	23	0.05	ND	ND	81	10	9	ND	32	2.43	4.7	0.04	0.33

[a] Abbreviations: I, Israeli soils; M, central Mexican soils; BCS, Baja California Sur soils; ND, not determined.
[b] Noncultivated soil.
[c] Cultivated soil.

previously described (5, 8). The final bacterial concentrations were 1×10^6 CFU/g of soil either with or without plants in Israeli soils, 1.77×10^7 CFU/g of soil for both soil and plant inoculation in Baja California soils, and 4.2×10^4 (to inoculate plants) or 1.44×10^8 (to inoculate soil) CFU/g in central Mexico soils. The inoculation level for soils containing plants was reduced to avoid damage to the plants caused by high inoculum concentrations (5). Soil was directly inoculated by adding a triply washed bacterial suspension in sterile water to each pot. Plants were inoculated at sowing as follows. Seeds were dipped for 5 min in the bacterial suspension under a vacuum of 600 mm Hg (ca. 80 kPa). Next, the vacuum was released abruptly, allowing the bacteria to penetrate the seed cavities which were previously filled with air (45). Each seed was sown with sterile tweezers to a depth of approximately 0.5 cm in the soil, prewetted to water field capacity.

Soil analyses and nomenclature. A total of 23 soil types were collected from various regions in Israel and Mexico and kept in hermetically sealed plastic containers at 4 ± 1°C. All soil samples were collected by commercial or experimental core samplers from the soil layer at a depth of 20 to 30 cm after the topsoil had been discarded (19). No attempt was made to preserve the soil intact; therefore, all samples should be considered disturbed. Several soil samples were from cultivated areas, and the others were from uncultivated land. Soil samples were collected from arid (<200 mm of rainfall per year) (11 soils; no. 10 to 20), semiarid (400 to 600 mm of rainfall per year) (5 soils; no. 5 to 9), mountainous (500 to 800 mm of rainfall per year) (6 soils; no. 1 to 4, 21, and 22), and tropical (>1,800 mm of rainfall per year) (1 soil; no. 23) areas (Table 1).

The following physical and chemical characteristics of each soil type were determined by standard soil analysis methods: soil texture and organic matter (47); pH; water field capacity and electric conductivity (20); nitrogen, phosphorus, and potassium content (56); and $CaCO_3$ content (29). The soils were classified according to the closest name in American nomenclature (Israeli soils) (46) or according to the Food and Agriculture Organization nomenclature (Mexican soils) (Table 1).

Soil sterilization. To avoid competition with native microorganisms and possible complications in the final analysis of abiotic soil factors affecting survival, all soils were steam sterilized by a standard procedure. First, each soil sample was heated for 1 h at 15 lb/in^2 in an autoclave. After being cooled, the soil was incubated for 24 h at 30 ± 1°C. This procedure was repeated three times. The contamination level after this sterilization procedure was zero as determined by the plate count method on nutrient agar plates. Nonsterilized soil samples were kept undisturbed in large plastic tubes (5 to 20 cm in diameter, 30 to 50 cm long) while they were being collected from the field. Comparisons of survival in sterile and nonsterile soil samples were made for five soil types from Israel representing different regions and types of soil: Mediterranean brown forest soil, brown basaltic soil, brown-red sandy soil, brown alluvial soil, and brown desert skeletal soil (Table 1).

Plant growth conditions. Plants were grown in 500-ml black 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 similar 250-ml pots. Plants were grown in a growth chamber at 25 ± 1°C at a light intensity of 100 mol/m^2/s and 60% ± 2% relative humidity (Baja California Sur and central Mexico soils) or in a fully controlled greenhouse at 22 ± 2°C and 60% ± 10% relative humidity (Israeli soils). Plants were irrigated every week with 5 to 15 ml of sterile, distilled water to avoid saturation as required according to the different sizes of the growing plants. Plants were fertilized once a week with 5 ml of half-strength Hoagland's solution.

Sampling and bacterial counts from soil and root samples. Samples (2 g of soil or approximately 0.5 to 1.0 mg [fresh weight] of roots and the adhering soil particles) were taken at each sampling. For sampling uniformity, soil from all 23 soil types was divided into two categories: soil not directly influenced by plant roots (bulk soil) and soil directly affected by roots (rhizosphere soil) (55). Therefore, rhizosphere bacteria were considered the bacteria that colonize the roots and the adhering soil particles. The samples were lightly sonicated at 25 W for 5 min (series 4710 sonicator; Cole Parmer, Chicago, Ill.) and then decimally di-

luted in 0.06 M phosphate buffer, pH 7.0, a harmless level for *A. brasilense* (45). The sonication was not strong enough to release all the bacteria attached to the roots and soil particles; thus, the released numbers should be considered minimal values. Bacteria from the sterile soils were counted after similar treatment by a conventional plate count method on nutrient agar (soils from Baja California, Mexico) or on N-free NFb medium (8) (soils from central Mexico), by indirect enzyme-linked immunosorbent assay (ELISA) (soils from Israel) (33, 35), or by the time-limited liquid enrichment technique (17) when the number of bacteria fell below the level of detection by the ELISA ($<10^4$ CFU per sample). Bacteria in nonsterile soils were counted by indirect ELISA (33, 35). All these methods are standard for *Azospirillum* counting and provide comparable results (8). Although strains Cd and Sp-245 originated from two different countries (the United States and Brazil, respectively), their survival rates in the seven arid soils of Mexico were similar. Differences in population levels between the two strains were indistinguishable by one-way analysis of variance ($P \leq 0.27$) after 45 days. On the basis of these results, data obtained in other experiments with these two bacterial strains were grouped together for analyses throughout the entire study.

Plant removal and soil percolation with water. We measured the survival of *A. brasilense* in soils (samples 1, 5, and 10) in which 6-week-old inoculated wheat plants were previously grown and removed. At this stage, the roots had filled the entire volume of the pots. Before the plants were removed, the soils were irrigated until saturation. The soils were dried at ambient temperature and sieved through a screen (pore size, 1 mm) before they were returned to the original pots. Sieving removed most major roots or root parts from the soils, but it left the pots without the continuous supply of root exudates. Control pots contained plants throughout the experimental period and received no second inoculation.

The percolation treatment of soils was done with similar types of soil after the plants were removed. The soils were extracted from the pots, dried, sieved to remove all root material, and loosely packed in large plastic columns (50 by 10 cm) (10). Next, each soil sample was slowly rinsed overnight with 10 liters of sterile, distilled water per column at 4 ± 1°C. After extraction from the columns, the soils were dried at ambient temperature (28 to 33°C), transferred back to the pots, and reinoculated. Controls for this experiment were nonpercolated soils from which the plants were removed, soil from pots containing growing plants, or plant-free soil.

AS production and survival tests. Soil is an extremely difficult environment to simulate (55). Nevertheless, to evaluate whether major abiotic soil parameters influence the survival of *A. brasilense*, two AS were created which resembled two soils from Israel, Terra Rosa soil (Rhodoxeralfs) and Hammada Soils of Mountains (Gypsiorthids). Compositions of both AS were based on thoroughly distilled water-washed quartz sand (fine, particle size of 0.02 to 0.2 mm; rough, particle size of 0.2 to 2 mm) and other components in the proportions described for the natural soils in Table 1. The two primary clay minerals in each soil were added in 1:2 (vol/vol) proportions to produce the required clay concentrations (montmorillonite and kaolinite for Rhodoxeralfs and montmorillonite and calcite for Gypsiorthids). Calcium was added as analytical $CaCO_3$, and organic matter was added as fine (60-mesh) sawdust. The water-holding capacity was adjusted by the addition of very fine vermiculite (particle size, <1 mm). Silt with an average particle size of 0.02 mm was donated by the local aquaculture industry. The pH was adjusted with phosphate buffer, pH 7 to 7.4 (which served also as a supply of K and P), and the nitrogen content was adjusted by adding NH_4NO_3. No microelements were added since they were probably present as scant contaminants in the analytical-grade reagents used. All the ingredients were mixed in a miniature, homemade soil mixer. Analysis of the AS revealed that their basic physical and chemical characteristics were very similar to those of the original soils. Soil moisture retention curves were compared and were also very similar. Portions of these mixtures (100 ml) were placed in black plastic pots and irrigated to water-holding capacity with deionized water. One half of the pots were planted with wheat plants, and the other half were kept unplanted.

Experimental design and statistical analysis. All experiments were carried out with three to four replicates per treatment. *A. brasilense* populations were determined at inoculation time and at intervals of approximately 1, 4, 14, 28, 35, and 42 days thereafter. Each sample was serially diluted. The *A. brasilense* population was determined with three replicates of each dilution. Since this type of study creates an excessive amount of data (more than 3,000 single determinations in this study), it was necessary to preprocess the data prior to the final analysis. This was done as follows. The rate of growth or death of the bacterial population was calculated according to Krebs' logistic equation of growth (31), $N_t = K/(1 + be^{-rt})$, where N_t is the size of the population at time t, r is the rate of growth, K is the maximum number that a population can achieve, and be is the natural log of $(K/N_0) - 1$, where N_0 is the initial number of bacteria in the culture. First, the rate of growth or death of the bacteria in each of the soils was correlated with the value obtained for each soil parameter in 23 different types of soil by using linear and multiple regression analyses at $P \leq 0.01$ and $P \leq 0.05$. Next, survival data for all the soils and all the soil parameters were analyzed by principal component analysis (PCA) (37). In PCA, each axis corresponds to an eigenvalue of the matrix or the variance accounted for by that axis. This analysis can reveal whether there is a general relationship (either positive or negative) between soil parameters measured in many soils and the rate of survival of bacteria in these soils. The analysis evaluates how much each variable affects the phenomenon. The closer the variables appear in the final analysis (see Fig. 3), the more related those variables are. The PCA standardized the data and eliminated differences in the measured range of each soil parameter. It also provided information on whether measured parameters acted together to affect bacterial survival when analysis of a single soil parameter had a nonsignificant effect. Both types of analysis were done with Statgraphics software (Manugistics, Rockville, Md.).

For statistical analysis in other experiments, we used one-way analysis of variance followed by Tukey's Studentized range test and the least-significant-difference test at $P \leq 0.05$ and Student's t test at $P \leq 0.05$ (40). For simplicity in the graphic presentations, standard deviations are not drawn and the average standard deviations of the lines exhibiting similar trends are given in the figure legends.

RESULTS

Survival of *A. brasilense* in the bulk soil and rhizosphere soil of 23 soil types. In general, *A. brasilense* Cd populations declined with time in all soils tested. This trend was similar for both nonsterile and sterile soils from the same origin. Protozoan predation and competition with other microorganisms were not evaluated. However, in all cases (which were compared by Student's t test at $P \leq 0.05$), the standard deviations for each sampling time overlapped for sterile and nonsterile soils. This statistical fact allowed us to simplify further experiments, and only sterile soils were analyzed in the rest of the study. Naturally, these soils became contaminated with time, possibly from airborne contaminants in the growth chamber, but generally, the colonization level of contaminants (routinely checked) was always significantly lower than the inoculated bacterial population in the soil and was never higher than 10^3 CFU/g of soil.

The survival rates of both inoculated *A. brasilense* strains differed significantly in the plantless bulk soil and in the rhizosphere of wheat and tomato plants growing in all soil types. In all the rhizospheres tested, viable cell numbers of both strains did not decline significantly, regardless of soil type, geographical origin of the soil, or the amount of rainfall each soil type received prior to sampling. Sometimes, viable cells proliferated above the original inoculation level. The levels of rhizosphere colonization were approximately 10^6 to 10^7 CFU per plant in arid and semiarid soils of Israel and Baja California and approximately 10^7 to 10^8 CFU per plant in the tropical or mountain soils of central mainland Mexico (Fig. 1 and 2; Rhizosphere symbols).

In the absence of plants, the general survival characteristics of *A. brasilense* differed greatly according to the geographical origin of the soils but not according to the original aridity of these soils. In Israeli soils, whether arid, semiarid, or of mountain origin (Fig. 1), the population rapidly declined with time and was not detectable within 35 days after inoculation. In contrast, *A. brasilense* populations in the arid soils of Baja California remained stable or even gradually increased over the 45-day period (Fig. 2A and B; "S" symbols). In the tropical and mountain soils of central mainland Mexico, the bacterial populations gradually decreased with time (Fig. 2C and D; "S" symbols). However, the decline was slower than it was in the Israeli soils, and a significant number of bacteria were detectable 45 days after inoculation.

Soil factors affecting the survival of *A. brasilense*. Of all the 15 physical and chemical parameters of the 23 soil types tested, only the levels of $CaCO_3$ and the number of viable cells of *A. brasilense* were negatively correlated. As the percentage of $CaCO_3$ increased, the survival rate decreased ($Y = -0.0264$ to 0.081, $r = 0.86$; significant at $P \leq 0.05$ by linear regression analysis). In 13 Israeli soils, the quantity of rough sand in soil had a negative effect much like the effect of $CaCO_3$ ($Y = -0.0235$ to 0.4675, $r = 0.82$; significant at $P \leq 0.05$).

A more comprehensive view of the effect of soil parameters

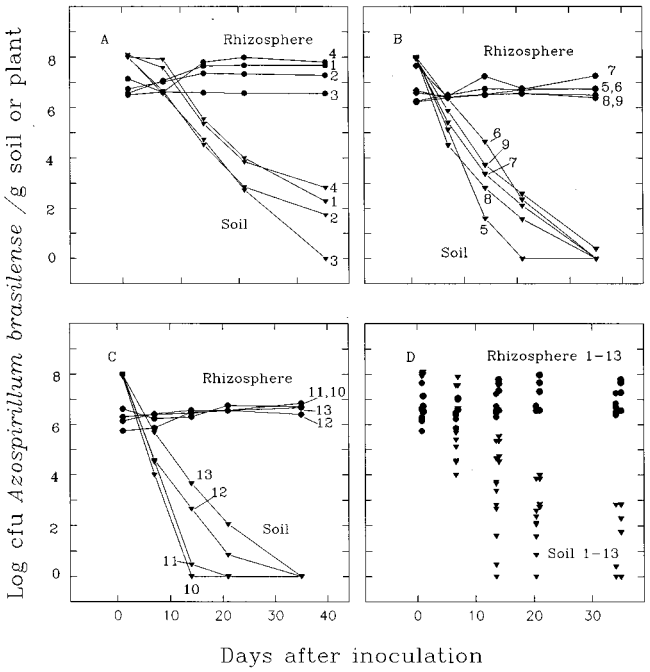

FIG. 1. Survival of *A. brasilense* in the soil and rhizosphere of samples of 13 soil types obtained from Israel (A through C) and general evaluation of survival of *A. brasilense* in Israeli soils (D). ●, survival in the rhizosphere; ▼, survival in the soil. Each datum point represents the mean for three to five independent samplings from pots, each conducted in triplicate. Numbers in each panel represent the serial numbers of the soil types described in Table 1. To simplify the complex graphs, the standard deviations were not drawn and are as follows: soil types 1 to 4 (A), rhizosphere (R) = 1.4851 and soil (S) = 1.5275; soil types 5 to 9 (B), R = 1.39024 and S = 1.5149; soil types 10 to 13 (C), R = 1.24098 and S = 1.5127.

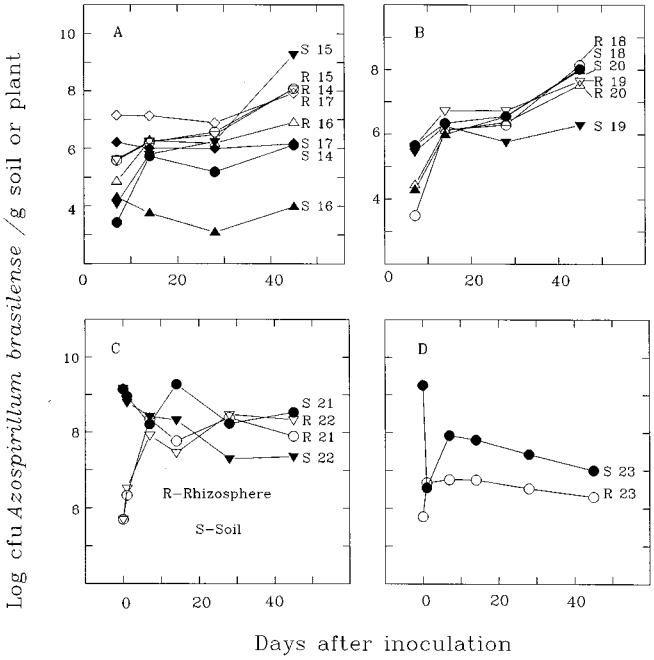

FIG. 2. Survival of *A. brasilense* in the soil and rhizosphere of samples of 10 soil types obtained from Baja California Sur, Mexico (A and B), central mainland Mexico (C), and tropical Mexico (D). Empty symbols, survival in the rhizosphere (R); solid symbols, survival in the soil (S). Each datum point represents the mean for three to five independent samplings from pots, each conducted in triplicate. Numbers in each panel represent the serial numbers of the soil types described in Table 1. To simplify the complex graphs, the standard deviations were not drawn and are as follows: soil types 14 to 17 (A) and 18 to 20 (B), R = 1.901 and S = 1.139; soil types 21 to 23 (C), R = 1.59906 and S = 1.7612.

on survival rate was provided by a PCA. Of 12 statistical components created and evaluated in this study, 4 accounted for about 90% of the variance detected in this study (Table 2). Therefore, only these components were plotted against each other. Two different trends were revealed by this analysis. Four soil parameters (percentages of clay, nitrogen, organic matter, and water-holding capacity) were positively associated with the survival of bacteria, i.e., they were grouped together in the positive part of the first component, whether we plotted component 1 versus component 2 or versus component 3 (Fig. 3). Another three soil parameters (percentages of $CaCO_3$ and fine and rough sand) were also grouped together, but in the negative part of the first component, indicating a negative effect on survival of *A. brasilense* in the soils (Fig. 3). The other six soil parameters (percentage of silt, pH, conductivity, percentages of K and P, and C/N ratio) had no apparent effect on survival of the bacteria in the soil.

Survival of *A. brasilense* in soils which previously contained plants (remnant soils). Removal of plants growing in three different types of soil from Israel (mountain, coastal-sandy, and desert) greatly affected the survival of *A. brasilense* in these soils. For 15 days after removal of the plants, the bacterial populations increased to a level similar to those in the rhizosphere of growing plants, but afterwards populations decreased sharply, reaching undetectable levels in all soils 90 days after inoculation (Fig. 4).

Survival of *A. brasilense* in water-percolated soils after plant removal. We compared the survival of the bacteria in remnant soils (mountain, coastal-sandy, and desert) from which plants were removed with the survival in similar soils from which plants had been removed but which were further percolated with water. Percolation of the soils had a diminishing effect on the *A. brasilense* population, which almost disappeared from the three soils 30 days after inoculation; numbers were undetectable after 60 days, even by the limited-enrichment method, which routinely detected less than 100 cells per sample (Fig. 5A). In two remnant soils, small numbers of bacteria (10^2 to 10^3 CFU/g of soil) were detected even 60 days after inoculation. Inoculation of soils with *A. brasilense* after water percolation had a diminishing effect on the level of nitrogen in the soils (Fig. 5B). However, water percolation and inoculation

TABLE 2. PCA of soil parameters versus survival of *A. brasilense* in 23 soil types

Statistical component	% Variance	Cumulative %
1	44.45	44.45
2	24.25	68.70
3	11.99	80.69
4	9.46	90.15
5	4.67	94.82
6	3.23	98.05
7	0.9	98.95
8	0.54	99.5
9	0.40	99.91
10	0.06	99.97
11	0.02	99.99
12	0.006	100.00

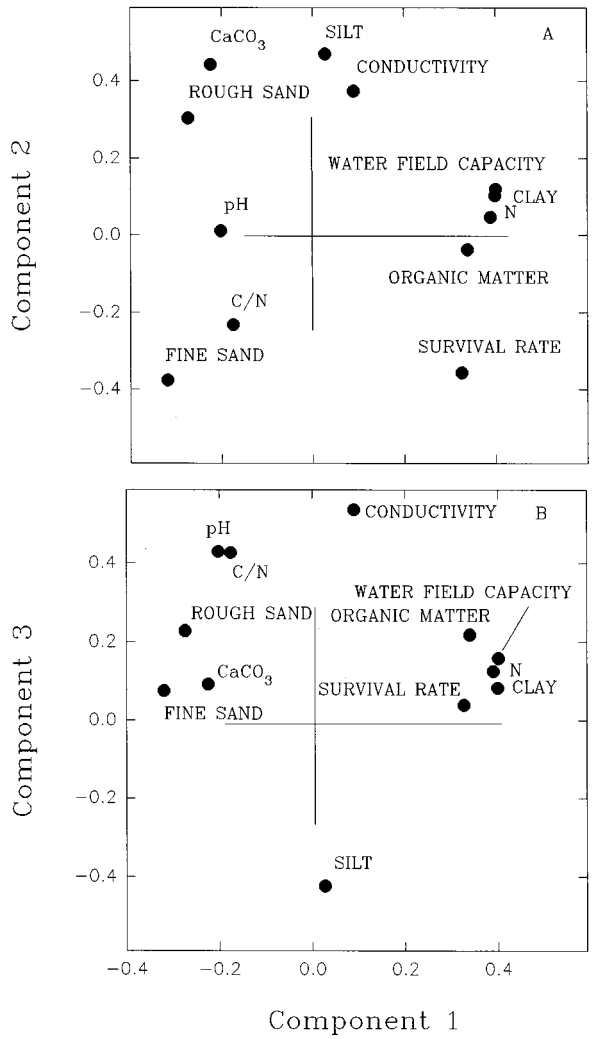

FIG. 3. PCA of 12 soil parameters in relation to survival of *A. brasilense* in 23 soil types. (A) Analysis of components 1 and 2 (which account for 69% of all the variations). (B) Analysis of components 1 and 3 (56% of all the variations). The central cross symbolizes the *x* and *y* axes and is presented only to illustrate the analysis. Numbers on both axes represent the estimated variability calculated by PCA and are not actual physical or chemical measurements.

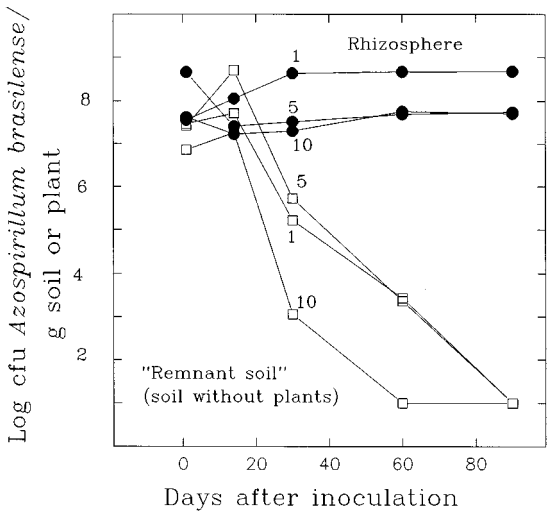

FIG. 4. Survival of *A. brasilense* in remnant soils, which previously contained plants (□), and in the rhizosphere. (●). Each datum point represents the mean for three to five independent samplings from pots, each conducted in triplicate. Numbers represent the serial numbers of the soil types described in Table 1. For simplicity, the standard deviations were not drawn and are as follows: rhizosphere, 1.4194; soil, 1.5216.

had only a slight effect on the level of organic matter in these soils (Fig. 5C).

Comparison of *A. brasilense* survival rates in natural soils and AS containing similar major components. Numbers of viable *A. brasilense* cells were almost identical in two natural soils (mountain and desert soils from Israel) and in two AS with the same major soil parameters (Fig. 6). The number of bacterial cells was constant, and numbers were similar in the rhizospheres of both natural soil and AS and rapidly declined in the absence of plants, reaching undetectable numbers in the desert soil after 30 days (Fig. 6).

DISCUSSION

The release of *Azospirillum* spp. into soils has a long history of unpredictable and 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, despite the ability of the bacteria to move towards and along the growing roots (10, 13, 30, 39). The general decline of introduced bacterial numbers in the soil often hampered the effectiveness of bacterial inoculation (55). Survival of plant-growth-promoting rhizobacteria in the soil is crucial because seed inoculation is impractical in many field applications (for perennial plants, vegetatively propagated plants, and trees or when more than one inoculation per season is required).

Several factors complicate the collection of sufficient data on bacterial survival in soil. (i) Every site may inherently be different, even on a microbial scale (54), and in many countries, even the basic soil composition is unknown or poorly understood. (ii) Few studies of the types and distribution of microenvironments in a particular soil have been done, despite the fact that these are widely recognized as being crucial to bacterial survival. (iii) Thorough studies of soil factors affecting survival of beneficial bacteria are scarce in the scientific literature (28). (iv) The inoculation industry has ignored the importance of basic ecological factors controlling inoculation (commercial *Azospirillum* inoculation technology is a good example of this [24, 42]). Thus, the principal aim of this study was to address the issue of survival of *Azospirillum* spp. in soil by creating sufficient data for future modeling and prediction of bacterial behavior in any given soil without the laborious studies of bacterial survival in every field.

Since *Azospirillum* strains were isolated from diverse geographical regions from tropical to temperate zones (6), temperature alone is unlikely to be the major limiting factor on the proliferation of native strains. Furthermore, as *A. brasilense* is apparently a nonspecific bacterium capable of colonizing numerous plant species (9), the growth of a particular plant species is probably not a requirement for survival. What was left, then, was the gamut of abiotic soil parameters and the biotic factors which were outside the scope of this study.

To verify the influence of abiotic factors on bacterial survival, we collected samples of 23 soil types representing different climatological conditions from tropical to arid zones and compared them in identical experiments using common strains of *A. brasilense*. All of these soils had been previously used for

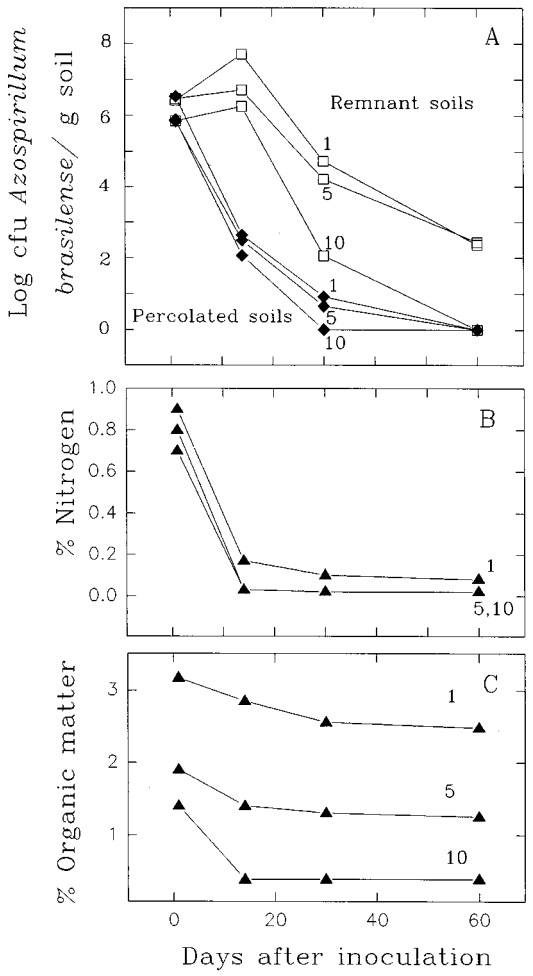

FIG. 5. (A) Survival of *A. brasilense* in remnant soils (□) and in soils percolated with water after plant removal (◆). (B and C) Levels of nitrogen (B) and organic matter (C) during the survival experiment. Each datum point represents the mean for three to five independent samplings from pots, each conducted in triplicate. Numbers represent the serial numbers of the soil types described in Table 1. For simplicity, the standard deviations were not drawn and are as follows: remnant soil, 1.6425; percolated soil, 1.435.

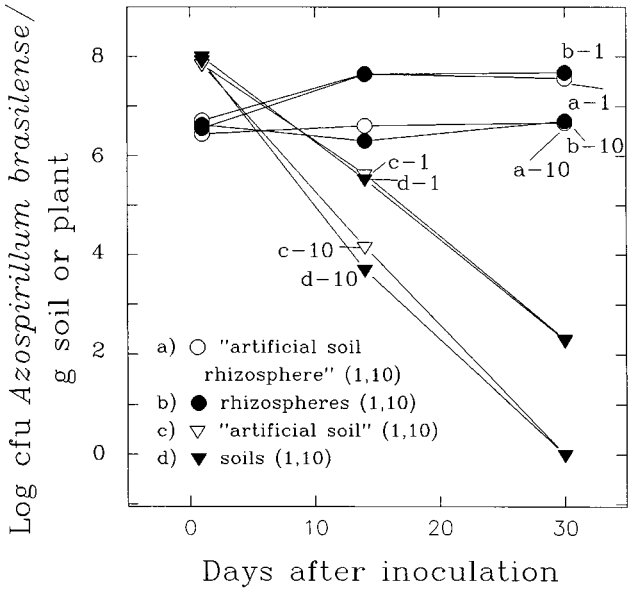

FIG. 6. Comparison of *A. brasilense* survival rates in natural soils and AS with similar major physical properties and chemical components. Each datum point represents the mean for three to five independent samplings from pots, each conducted in triplicate. Numbers represent the serial numbers of the soil types described in Table 1. For simplicity, the standard deviations were not drawn and are as follows: natural soils, rhizosphere = 1.42 and soil = 1.52; AS, rhizosphere = 2.4164 and soil = 2.5156.

Azospirillum experiments, and in all soils, improvement of some plant parameters occurred by inoculation (6a).

It was clear, even without the aid of any statistical analysis, that *A. brasilense* was a typical rhizosphere bacterium. Large numbers of viable cells were found in the vicinity of roots, regardless of soil characteristics and as long as plants were growing in these soils. In the absence of plants, survival rates differed significantly, being more related to the geographic than to the climatological origin of the soils. In the arid soils of Israel, *A. brasilense* survived poorly, while it proliferated in the arid soils of Baja California. Using statistical analyses of the bacterial survival data and the soil parameters, 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 correlated with bacterial survival. No other single parameter was responsible by itself or was a major factor affecting survival. However, PCA revealed that when several factors were grouped together (percentages of clay, nitrogen, organic matter, and water-holding capacity), they positively affected survival, whereas the levels of $CaCO_3$ and the rough sand or fine sand in the soil negatively influenced the survival of the bacteria.

The effects of single soil parameters such as soil texture and clay content on the survival and proliferation of soil and rhizosphere bacteria are known (25, 54, 55). The novelty of this study is that only the combined action of several lesser parameters was found to significantly determine the survival of *Azospirillum* spp. in sterile soil. Nevertheless, these parameters were unimportant with plants growing in the soils. In sum, the seven abiotic soil parameters acting together on the bacteria determined the survivability of *A. brasilense* in the soil. The proportional effects of each abiotic parameter on the overall effect are not known, nor is it known whether manipulation of the soil parameters can alter bacterial survival. Biotic parameters, like protozoan predation (which may decimate the population of the introduced bacteria), were not determined in this study. The very low level of contamination that developed at the later stages of experiments with sterile soils probably had little effect on *A. brasilense* populations.

Apparently, other soil abiotic parameters (microelements and soil pore space) have little, if any, effect on the general survival of *A. brasilense* in sterile soil. When AS were created, only the 15 major, nonbiological soil parameters were simulated. However, the population level of *A. brasilense* in these AS was almost identical to those in the original soils. Nevertheless, these results concur with the ability of *Azospirillum* spp. to grow in the absence of any particular microelement (43) and with the fact that only a small fraction of the soil pore space is occupied by bacteria (44).

Despite the clear effect of plants on the survival of *A. brasilense*, one should consider the long-term residual effect of plants on soil composition (38). It is widely accepted that plant roots represent the major source of carbon and nitrogen for soil. Both soluble compounds (like root exudates) and insoluble compounds (like remnants of root cortex cells and, later,

products of the decomposition of small roots) are released into the rhizosphere and, later, into the soil bulk (55). This study demonstrated that after removal of plants from the soil, the remaining residual materials in the soil supported *A. brasilense* for a few weeks. Thereafter, when these nutrients presumably were exhausted, the other abiotic soil parameters, probably with the aid of the biological interactions in the rhizosphere, determined the survival rate of the bacteria. This conclusion was supported by the finding that when soils were sieved and percolated with water after the plants were removed, the bacterial population decreased rapidly and drastically.

In conclusion, we propose that *A. brasilense* is a rhizosphere colonizer whose survivability is independent of soil aridity. Seven abiotic soil parameters (percentages of clay, nitrogen, organic matter, $CaCO_3$, fine and rough sand, and water-holding capacity) control the survival of this bacterium in plantless soils. Disturbance of the soil (by water percolation or plant removal) directly and rapidly affects the *A. brasilense* population levels.

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