The fate of field-inoculated *Azospirillum brasilense* Cd in wheat rhizosphere during the growing season

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Following inoculation of wheat grown under field conditions with *Azospirillum brasilense* Cd, the bacterial population in the plants rhizosphere during the winter growth season was monitored. Seven field experiments were conducted during 3 years, in five soil types, and the bacteria were counted using two different methods of enumeration. *Azospirillum brasilense* Cd were detected in the wheat fields up to 105 days after inoculation at a level of 10^4 to 5×10^5 cells/g fresh weight roots, with a very small variation between colonized plants, regardless of the inoculation method. Only a small negligible decrease in the bacterial population occurred during the coldest period of the growing season. Later, the population increased again to the initial level and remained constant. Although inoculated twice, a part of the plant population had not been colonized by *A. brasilense* Cd. We propose that *A. brasilense* Cd colonized, although in relatively small numbers, the root system of wheat during the winter season, under suboptimal temperatures for this bacterial species.

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Croissant sous des conditions champêtres, des blés ont été inoculés avec de l'*Azospirillum brasilense* Cd et le comportement de la population bactérienne a été suivi au niveau de la rhizosphère de ces plantes durant la saison de croissance hivernale. Sept expériences de plein champ, comportant cinq types de sols, ont été menées sur une période de 3 ans et les bactéries ont été dénombrées selon deux méthodes d'énumération. Jusqu'à 105 jours après inoculations, les *A. brasilense* Cd ont été décelées à des niveaux de 10^4 à 5×10^5 cellules/g de poids frais des racines, avec peu de variation entre les plantes colonisées et, ce, sans égard à la méthode d'inoculation. Une diminution négligeable de la population bactérienne est survenue au cours de la période la plus froide de la saison de croissance; ultérieurement, un regain de population a atteint le niveau initial et celui-ci est demeuré constant. Malgré une double inoculation, une partie de la population de blés n'a pas été colonisée. Il s'avère donc, que l'*A. brasilense* Cd colonise, bien qu'à un degré relativement faible, le systeme racinaire du blé au cours de la saison hivernale et, ce, dans des conditions suboptimales de température pour cette espèce de bactérie.

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Introduction

Inoculation of crop plants with beneficial rhizosphere bacteria is being tested worldwide owing to their potential contribution to plant productivity (Millet et al. 1984; Rennie and Larson 1979; Reynders and Vlassak 1982; Smith et al. 1984). Root colonization by the bacteria is essential in the cascade of events which ends in the contribution of the bacteria to plant growth and yield (Suslow 1982). However, factors such as limited migration of the benificial bacteria in soil, adsorption of bacteria to soil particles, and competition with the natural populations of rhizosphere bacteria for nutrients leaking from the roots decrease the size of the bacterial population and in turn their positive effects on plant growth. (Albrecht et al. 1983; Bashan 1986*d*; Bashan et al. 1987; Hubbell and Gaskins 1984).

A major difficulty encountered in the use of *Azospirillum* is the inconsistant results often reported (Albrecht et al. 1983; Bashan 1986*a*; Okon 1985; Smith et al. 1984): significant increases in yield occurred in tandem with negligible or even reduced yields. Some of this variability maybe due to failure of the inoculated organism to establish itself on or inside roots; high percentage of wheat roots fail to be colonized by the applied bacteria (Bashan 1986*b*, 1986*c*).

Enumeration of the established population of the applied bacteria in the rhizosphere during the growing seasons is important for understanding the colonization process. Reported numbers range from 10^1 to 10^2 colony-forming units (cfu) per g of roots (Smith et al. 1984) to over 10^8 cfu/g (Baldani et al. 1983, 1986). The population size of the applied bacteria was determined usually once or twice in the course of field experiments (Baldani et al. 1983, 1986; Nur et al. 1980), but interval monitoring, especially of *Azospirillum*, during the growing season is rare (Albrecht et al. 1983; Marocco et al. 1983).

In this study the dynamics of the rhizosphere population of *A. brasilense* Cd was monitored after inoculation during the wheat-growing season in several soil types under field conditions.

Materials and methods

Organisms

Azospirillum brasilense Cd (ATCC 29710) was inoculated onto wheat plants (*Triticum aestivum cv.* Deganit) during the 3 years of the field experiments.

Bacterial inoculants and details of the field experiments

Bacterial peat inoculant was prepared according to Thomson (1980) as follows: to prepare 100 g of inoculant, 45 g grounded fine peat (40 mesh, Nitragin, Milwaukee, U.S.A.) was thoroughly mixed with 5 g $CaCO_3$ and 20 mL of tap water (final pH 6.8) in polyethylene bags sealed with cotton plugs. The bags were then γ-irradiated (2.5 mrad; 1 rad = 10 mGy). Bacteria were grown in nutrient broth (Difco) medium for 16 h at 30 ± 2°C in rotary shaker (250 rpm). Thirty millilitres of the bacterial culture (approximately 5×10^9 cfu/mL) were aseptically added to each bag, mixed, and incubated for 7 days at 30 ± 2°C. Every 2 days the peat was mixed by shaking the bags. The final minimal number of bacteria in the inoculant, determined by the plate count methods, ranged from 5×10^7 to 5×10^8 cfu/g inoculant. The bags were stored up to additional 7 days at 4 ± 1°C. One day before plant

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inoculation, the bags were transferred to 30 ± 2°C. Bacteria release from the inoculants after application to plants varied and ranged between 10^3–10^5 cfu/g per day.

Alternatively, soils were inoculated with dry synthetic alginate beads (2 mm in diameter) composed of sodium alginate (BDH), skim milk (Difco), and bacterial culture (Bashan 1986*b*). These beads contained 10^8–10^9 cfu/g dry beads, and were designed to release 10^6 cfu/g per day. In addition to these application methods, the pot experiment was inoculated also with 10^6 cfu/mL of twice-washed, 18-h-old, bacterial suspension per 1 mL of soil (Bashan 1986*a*). All plant samples analyzed in this study were collected during the winter season which is the growing season of wheat in Israel (sowing in November and harvesting in June). All the technical data concerning location of fields, soil types, inoculation and sampling dates, or other details are summarized in Table 1.

Pot experiment

The pot experiment was conducted outdoors during the 1985–1986 season as described elsewhere (Bashan 1986*a*) and briefly described in Table 2.

Plant samples

Each field experiment was sampled at 2- to 3-week intervals throughout the seasons as indicated in Table 1. Samples of 5–10 plants were obtained at random from each of five plots, using a large spade (20 × 30 cm). Samples of up to 4-week-old plants, containing most of the root systems, were packed with the original field soil. From older plants, a core of the root system was sampled up to 30 cm depth (Bashan and Wolowelsky 1987). These samples, consisting of roots and soil, were packed in polyethylene bags, incubated at 4 ± 1°C overnight, and analyzed for bacterial number. In Nir-Am experiment (expt. 7, 1985–1986), approximately 50 plants were sampled at 3- to 4-day intervals. In the pot experiment, all plants were sampled and analyzed.

Azospirillum brasilense *Cd counts from wheat roots*

Azospirillum brasilense Cd was enumerated by two methods. During the 1983–1984 season, roots of plants from the field were lightly rinsed under a gentle stream of water, homogenized first by a disperser (Ystral, x10/20, Federal Republic of Germany) and then by a fine glass homogenizer (Kontes, U.S.A.). After centrifugation (12 000 × *g*, 10 min), the pellet was dissolved in 2 mL of 0.06 M potassium phosphate buffer (pH 7.0), decimally diluted in the same buffer, and the dilutions were inoculated into nitrogen-free semisolid media (SS) for the most probable number (MPN) determination, and onto two semiselective (BL or BLCR) media as previously described (Bashan and Levanony 1985). Bacterial numbers were referred as cells per gram of fresh weight of roots (Postgate 1969).

During the 1984–1985 and 1985–1986 seasons, roots were sampled and treated as described above. *Azospirillum brasilense* Cd cells were detected, identified, and enumerated by the highly specific enzyme-linked immunosorbent assay (ELISA) developed for the detection and enumeration of this strain (Levanony et al. 1987) as follows: after centrifugation of root homogenates the pellet was dissolved in a minimal volume of 2–3 mL of coating buffer and was used to coat microtiter plates or for competition ELISA.

The incidence of successful colonization of plant roots was tested by random sampling of 25 plants at each sampling date. Each plant root system was placed into SS liquid medium for bacterial enrichment (Okon et al. 1977). Development of bands or turbidity after 48 h of incubation was scored. Specific identification of the developing bacteria in the bands as *A. brasilense* Cd was determined by pumping out the bands by Pasteur pipets, and the solutions were used as competitors in the competition ELISA tests. *Azospirillum brasilense* Cd presence was marked in each tube and the percentage of positive root colonization was determined.

Soil temperature

Data on soil temperature in each experimental area were compiled from the multiyear measurements taken by the Israeli meteoroligical service during the years 1964–1973. The data base consisted of observations of soil temperature at 10 cm depth measured at 0800, 1400 and 2000. Each month was divided into three periods: two periods of successive 10 days and a third period covering the remaining days of the month. There were at least 50 measurements in each observation period. Usually data are from 100 different observations (Zemel and Lomas 1977). These soil temperatures resembled well the actual soil temperature prevailing in the season of 1983–1984 and were calculated as the mean of the lowest and the highest temperature at a certain localization.

Experimental design

All field experiments were designed in block fashion with five to eight replicates. Samples were taken from each replicate as described earlier. Plots size varied from 1.2 × 6 m in 1985–1986 up to 8 × 20 m in other seasons. Since great variation existed in root colonization (Bashan 1986*a*, 1986*b*), the given data from the 1984–1985 and 1985–1986 experiments represent the actual numbers of bacteria in the samplings. Data of 1983–1984 experiments were measured in colonized plants only. Significance is given by standard error.

Results

Detection and enumeration of A. brasilense *Cd in wheat plants grown in pots 15 days after inoculation*

Wheat plants grown in pots were inoculated with *A. brasilense* Cd by four different techniques and root colonization was enumerated by two different methods. Regardless of the method of inoculation or enumeration, *A. brasilense* Cd was found to be able to colonize wheat roots. Application of bacterial liquid culture or peat inoculant on the soil surface resulted in a similar percentage and level of root colonization. Application of peat inoculant or alginate beads mixed with seeds at sowing increased slightly the level of root colonization. However, the percentage of colonized roots was significantly higher when alginate beads were used (Table 2).

The fate of A. brasilense *in the field during 1983–1984 experiments*

Colonization dynamics of *A. brasilense* in wheat roots were monitored in five field experiments. Regardless of the soil type, the *A. brasilense* level in wheat roots was similar in all experiments in the beginning of the season (10^4–10^5 cfu/g roots fresh weight). During the coldest period of the growing season, a small decrease in *A. brasilense* population was detected in most experiments, but the population increased concomitently with the increase in temperatures during March and April. The population size of bacterial cells per gram of roots did not increase during the season (Fig. 1 A– 1 I).

Variation in the colonization level of A. brasilense *Cd in the field during 1984–1985 experiments*

The population dynamics of *A. brasilense* Cd was monitored in samples obtained from the field by the specific competition ELISA (expt. 6). Two different phenomena were observed in the inoculated roots: (*i*) although inoculated twice, some of the plant roots obtained from the plots were not colonized by *A. brasilense* Cd throughout the growing season; (*ii*) colonization levels were similar in all the replicates and the variation between colonized plants was small and not significant (Fig. 2). The bacterial population survived for at least 105 days after inoculation on roots at a constant level (per gram of fresh weight of roots).

Presence of A. brasilense *Cd in wheat roots following inoculation by two methods during 1985–1986 season in heavy loess raw soil*

Azospirillum brasilense Cd was inoculated at sowing using

TABLE 1. List and details on the field samplings for

Expt. no.	Location	Soil type[b]	Inoculant type	Method of inoculant application	Amount of peat inoculant, g/m^2
1	Western Yizreel Valley (Mishmar HaEmek)	Colluvial–alluvial	Peat	Drip[d]	0.5
2	Eastern Yizreel Valley (Beit Alfa)	Brown alluvial	Peat	Drip	0.5
3	Northern Negev (Negba)	Alluvial	Peat	Drip	0.5
4	Northwestern Negev (Nir-Am)	Loess raw	Peat	Drip	0.5
5	Western Negev (Gvulot)	Loessial sandy	Peat	Drip	0.5
6	Eastern lower Galilee (Moledet)	Brown alluvial	Peat	Drip	2
7	Northwestern Negev (Nir-Am)	Loess raw	Peat or alginate beads[g]	Mixed[h]	5 or 4 beads per seed

[a]Day/month/year.
[b]According to Ravikovitch (1981).
[c]Plants were inoculated immediately after seedling emergence (except for expt. 7).
[d]Drip, a suspension of bacterial liquid inoculant was dripped on the wet soil surface after seedling emergence. According to Millet *et al.* (1984).
[e]MPN, MPN method in nitrogen-free semisolid medium and colony formation on semiselective medium. According to Bashan and Levanony (1985).
[f]According to Levanony *et al.* (1987).
[g]According to Bashan (1986*b*).
[h]Mixed, peat inoculant or beads were mixed with seeds and sown simultaneously in dry soil. After sowing, the field was irrigated at 400 m^3 water/ha.
[i]No. of plants per inoculation treatment.

either peat inoculant or alginate beads mixed with the seeds. Samples were taken at 4-day intervals immediately after seedling emergence (expt. 7). *Azospirillum brasilense* Cd could be detected and identified in wheat roots, by the specific ELISA method, from the emergence day (8 days after sowing) until at least 70 days after sowing. Both methods of application resulted in wheat root colonization. The level of *A. brasilense* Cd in the root ranged from 5×10^4 cells/g fresh weight roots (15 days after sowing) to 1.25×10^4 cells/g fresh weight roots (22 days after sowing) and then slowly decreased below the sensitivity of the ELISA method. To obtain longer periods of qualitative detection of *A. brasilense* Cd population, bacteria in samples obtained from roots were first enriched in semisolid nitrogen-free medium for exactly 48 h and then identified by ELISA. Using this technique, *A. brasilense* Cd was detected up to 70 days after inoculation regardless of the inoculation method (Fig. 3).

Discussion

Inoculation of plants by beneficial associative rhizosphere bacteria of the genus *Azospirillum* is still in its experimental stage despite a decade of research. Positive responses of the inoculated plants are unpredictable (Gaskins et al. 1985; Patriquin et al. 1983; Schank and Smith 1984), and therefore this inoculation system is not yet commercially utilized. Postinoculation processes such as root colonization, bacterial survival, migration and fate of bacteria in the soil, and bacterial activity within the plant should be studied in order to understand the relationship between plants and beneficial bacteria. Most studies on bactrial inoculation showed changes in plant parameters after inoculation. These changes were referred directly to the inoculation effect, a matter that has not yet been sufficiently proven. Moreover, information about bacterial population dynamics during the growing seasons is rare (Albrecht et al. 1983; Marocco et al. 1983). Nearly all available data on *Azospirillum* numbers in the rhizosphere are based on most probable number (MPN) determinations and (or) on plate count methods or on countings from enrichment cultures. The first two methods can minimize the estimations of *Azospirillum* because members of this genus usually form massive microcolonies or aggregates on the root surfaces and inside them, and therefore each aggregate may be counted as one organism only (Bashan et al. 1986; Okon 1985; Okon et al. 1983). Moreover, other isolates resembling *Azospirillum* in features such as pigmentation, microaerophilic growth, N_2 fixation, acetylene reduction, and antibiotic resistance could be unappropriately scored also. On the other hand, the enrichment methods, especially those performed for a long incubation period, provide little information about the numerical abundance of the applied bacteria in the plant rhizosphere.

Since the bacterial enumeration methods in the rhizosphere are inaccurate, there is no consensus about the colonization level of the applied bacterial strain in the plant rhizosphere. Thus, bacterial levels in the different reports vary from very low (10^2 cfu/g roots) to high numbers (more than 10^8 cfu/g roots) (Albrecht et al. 1983; Baldani et al. 1983, 1986; Döbereiner and

enumeration of *A. brasilense* Cd in wheat rhizosphere

Sample size (plants)		Method of bacterial detection	Dates[a]			Interval (days)
No. of plants/plots	No.of plots/sampling		Inoculation[c]	First sampling	Last sampling	
5–10	5	MPN[e]	9/12/83	1/1/84	14/4/84	25
5–10	5	MPN	9/12/83	1/1/84	14/4/84	25
5–10	5	MPN	10/12/83	20/12/83	4/3/84	20
5–10	5	MPN	1/12/83	20/12/83	28/3/84	20
5–10	5	MPN	1/1/84	10/1/84	28/3/84	20
5–10	5	Indirect and competition ELISA[f]	15/1/85	28/1/85	21/4/85	15–20
50[i]		Indirect and competition ELISA	12/11/85	24/11/85	19/1/86	4–14

TABLE 2. Enumeration of *A. brasilense* Cd by various methods in roots of wheat plants grown in pots 15 days after inoculation

Method of bacterial inoculation	*A. brasilense*, cfu/g roots		% of plants colonized by bacteria
	Improved selection technique[a]	Competition ELISA[b]	
Culture suspension[c]	$7.4\pm0.8(\times10^4)$	$6.1\pm0.6(\times10^4)$	55±4
Peat inoculant	$6.6\pm1.1(\times10^4)$	$8.3\pm1.2(\times10^4)$	63±4
Surface application[d]	$1.4\pm0.4(\times10^5)$	$5.6\pm0.4(\times10^5)$	77±3
Mixed with seeds[e]			
Alginate beads[f]	$1.2\pm0.3(\times10^5)$	$4.8\pm0.6(\times10^5)$	94±2

NOTE: Plants were grown in 3 L of brown–red degrading sandy soil of Rehovot in a nethouse.
[a]According to Bashan and Levanony (1985).
[b]According to Levanony *et al.* (1987).
[c]Bacterial culture (18 h) at the logarithmic phase applied on soil surface at final concentration of 10^6 cfu/mL soil.
[d]Applied on soil surface at seedling emergence (2 g/m^2).
[e]Applied at sowing after mixing with seeds.
[f]Prepared according to Bashan (1986*b*) and applied at sowing (four beads/seed).

Baldani 1979; Germida 1985; Marocco et al. 1983; Nur et al. 1980; Okon et al. 1977, 1983), on a background of a total number of at least 10^8 cfu/g roots of N_2-fixing bacteria (McClung et al. 1983; Rennie et al. 1982).

The specific quantitative ELISA which was developed especially for the detection and enumeration of *A. brasilense* Cd, combined with the MPN method, enabled us to monitor the population dynamics of this strain in the field after inoculation without the interference of other bacteria naturally occuring in the rhizosphere in large numbers.

In these experiments, *A. brasilense* Cd was capable of colonizing wheat roots at the rates of 10^4–10^5 cells/g fresh weight. Despite the fact that 785 determinations were performed, no higher bacterial values were obtained. In addition, only a part of the plants in a given experimental plot were colonized by this strain, even after two bacterial applications. However, this level was stable throughout the growing season in most experiments. Therefore reports of a mush higher level of root colonization in the field by *Azospirillum* (Baldani et al. 1986) can not be confirmed by our results.

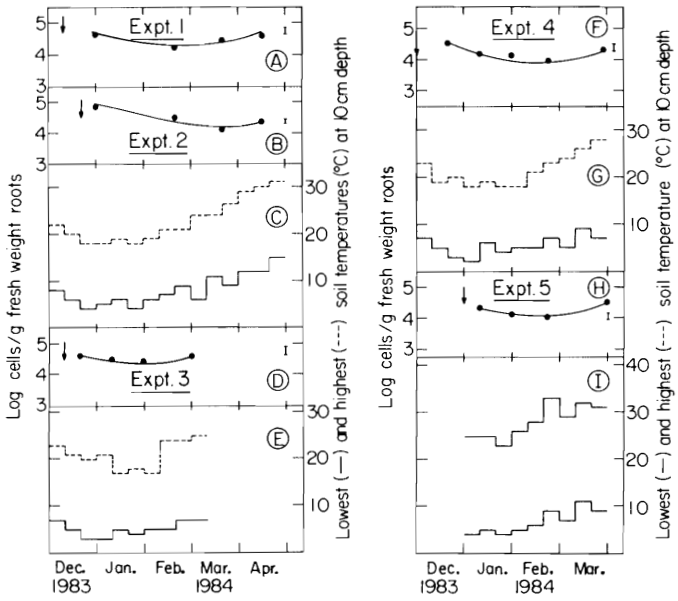

FIG. 1. Number of cfu of *A. brasilense* Cd in wheat rhizosphere in five different soil types in the 1983–1984 season. (A) Expt. 1; (B, C) expt. 2; (D, E) expt. 3; (F, G) expt. 4; (H, I) expt. 5. Arrows indicated the date of bacterial application and bars represent the standard error of the lines.

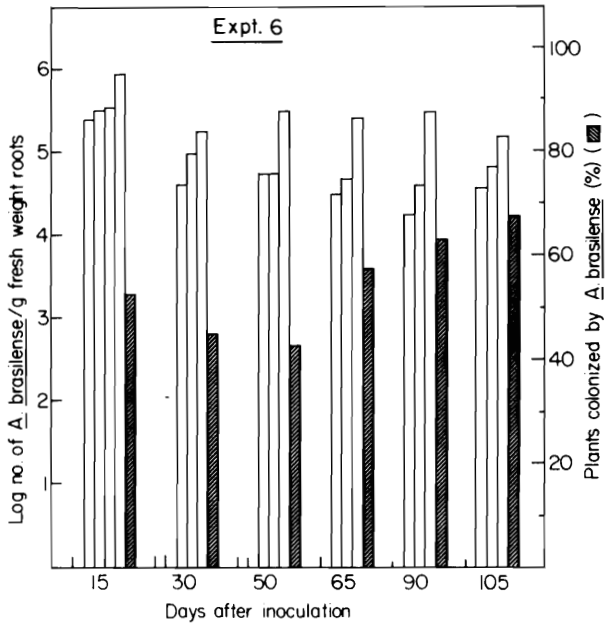

FIG. 2. Variation in colonization level and incidence of *A. brasilense* Cd in the field during 1984–1985 experiment (expt. 6) in brown alluvial soil. Each empty column represents counts of *A. brasilense* in a sample consisting of roots of five plants. Absence of a column indicates that *A. brasilense* was not detected in the sample. Hatched columns represent the percentage of plants colonized by *A. brasilense* calculated from 25 plants sampled each time.

Hubbel and Gaskins (1984) and Smith et al. (1984) showed that after inoculation with the same strain of *A. brasilense* (Cd), the population level of the bacteria in either pots or under field conditions was rapidly reduced to a very low level. Our results differ from these findings. These differences probably resulted from different experimental conditions such as soil types, plant species, or enumeration methods.

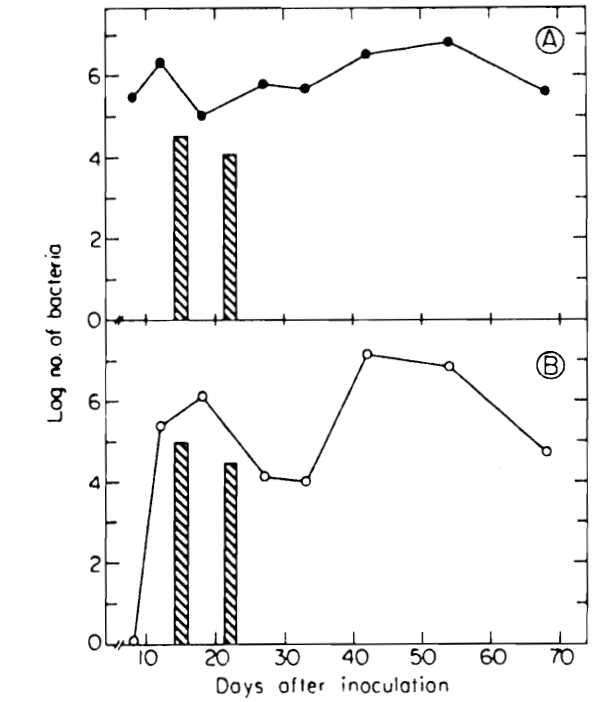

FIG. 3. Colonization level of *A. brasilense* Cd following two inoculation methods during 1985–1986 in heavy loess raw soil. (A) Peat inoculant; (B) alginate beads inoculant. ○, ●, Bacterial numbers (log no. of bacteria/mL) after limited enrichment in semisolid nitrogen-free medium. Histograms represent bacterial enumeration (log no. of bacteria/g fresh weight roots) by competition ELISA.

It should be emphasized that nearly all the young roots at a given bacteria-colonized plant were colonized to about the same extent, regardless of the root region which was sampled (Y. Bashan and H. Levanony, unpublished data). This mode of colonization indicated that this species is actually a good competitor in the wheat rhizosphere, and corroborates the opinion of Okon (1985), but is not in accordance with those of Albrecht et al. (1983), Germida (1986), and Smith et al. (1984).

Small changes in *Azospirillum* level in roots were only slightly related to seasonal variations in soil temperature. In the coldest period of the growing season a small reduction in the bacterial population was detected. The population overcame this and reached about its initial level per gram of roots when warmer soil temperatures prevailed at about the middle of the growing season (March and April). In no case were these changes significantly different. However, even the warmer temperatures did not reach the optimal growth temperatures in culture of this bacterial species (Okon et al. 1977).

Thus, according to the data presented in this study, it can be suggested that *A. brasilense* Cd can colonize wheat roots at a constant, moderate level. Only a fraction of the inoculated plant population were also colonized by the bacteria, but this colonization is hardly affected by the inoculation method, soil type, or by environmental changes occurring during the growing season in the field.

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