An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense*

YOAV BASHAN[1] AND HANNA LEVANONY

Department of Plant Genetics, The Weizman institute of Science, Rehovot 76 100, Israel

Accepted July 4, 1985

BASHAN, Y., and H. LEVANONY. 1985. An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense*. Can. J. Microbiol. **31**: 947–952.

An improved selection technique for isolation and enumeration of *Azospirillum brasilense* was developed. The technique is based on successive liquid enrichments in nitrogen-free semisolid medium supplemented with streptomycin, followed by the most probable number counting method and verification on a selective medium. The latter is based on Okon's nitrogen-free medium supplemented with cycloheximide (250 mg/L), streptomycin sulphate (200 mg/L), sodium deoxycholate (200 mg/L), 2,3,5-triphenyltetrazolium chloride (15 mg/L), and Congo red (1000 mg/L). This medium was found to be superior to other available diagnostic media. The technique was readily applied to detect and count *A. brasilense* Cd in inoculated wheat roots.

BASHAN, Y., et H. LEVANONY. 1985. An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense*. Can. J. Microbiol. **31**: 947–952.

Une technique améliorée de sélection a été développée pour l'isolement et le dénombrement de *Azospirillum brasilense*. Cette technique est basée sur une succession d'enrichissements liquides d'un milieu semi-solide dépourvu d'azote, mais additionné de streptomycine, suivie d'un comptage par la méthode du "Nombre le plus probable" et d'une vérification sur un milieu sélectif, soit celui de Okon. Ce milieu, libre d'azote, est additionné des substances suivantes: cycloheximide (250 mg/mL), sulfate de streptomycine (200 mg/L), désoxycholate de sodium (200 mg/L), 2,3,5-triphényl-chlorure de tétrazolium (15 mg/L), rouge de Congo (1000 mg/L). De tous les milieux de diagnostic disponibles, celui-ci s'est avéré supérieur. Cette technique fut appliquée avec facilité pour déceler et dénombrer l'*A. brasilense* Cd chez des racines de blé inoculées.

[Traduit par le journal]

Introduction

Increased interest has arisen in bacteria of the genus *Azospirillum* during the last few years mainly because of their possible contribution to the yield of various cereals (Reynders and Vlassak 1982; Kapulnik and Okon 1983; Millet and Feldman 1984). Most scientists inoculate with a local isolate of the genus, using a known strain such as *A. brasilense* Sp 7 or Cd as a reference bacteria for identification purposes.

The identification of a specific species of soil bacteria is usually a difficult and cumbersome task (Döbereiner and Day 1974; Tarrand et al. 1978; Schank et al. 1979; De-Polli et al. 1980; Rennie 1980, 1981), entailing either extensive biochemical, immunological, or DNA-hybridizations tests, or the recently developed computerized identification system. Previously proposed media for culturing N_2-fixing bacteria and isolation procedures for *Azospirillum* (Balandreau 1983; Döbereiner and Day 1974; Okon et al. 1977; Rennie 1981; Rodríguez Caćeres 1982) were not selective enough, enabling the development of many different types of bacteria, some of which are quite similar to those of *A. brasilense*. Therefore, the development of a selective medium, or a reliable isolation procedure, or both, was urgently needed.

In this study we report on a quick, simple, and efficient laboratory technique for isolation and enumeration of *A. brasilense*. The technique was also applied to bacteria isolated from inoculated wheat roots.

Materials and methods

Media

The media used in the course of developing the selective medium for *A. brasilense* are described in Table 1. The selective media (abbreviated in Table 1 as BL and BLCR) were developed by evaluating the inhibitory effects of various substances added separately to King-B medium (King et al. 1954) on the growth of *A. brasilense* Cd. These chemicals and their final concentration are described in Table 2. All chemicals were gamma irradiated (if necessary) at the Weizman Institute of Science, with 1 Mrad (1 rad = 10 mGy), using cobalt 60 source, and applied asceptically to the autoclaved medium.

Bacteria

Azospirillum brasilense strain Cd (ATCC 29710) was used as a model organism throughout the development of the diagnostic technique. The following bacteria were tested for their ability to grow on the BL and BLCR selective media: *A. brasilense* Cdw-1; *A. brasilense* Cdw-3 (azide and chloramphenicol mutants of Cd, respectively); *A. brasilense* FT-400, FT-326, and FT-339 (overproducing auxin mutants); *Azospirillum* sp.T1; and *Azospirillum* sp.T2 (isolated from roots of wild cereals that grow in Israel) as well as seven unidentified rhizosphere bacteria which were found to colonize well the roots of cultivated wheat.

Microaerophilic growth, acetylene reduction, and MPN determination

Tests for both microaerophilia and acetylene reduction were performed in tubes containing SSS medium. Microaerophilic growth was detected by the appearance of a bacterial band, 0.5 to 1.5 cm below the liquid surface. When such growth was observed, acetylene reduction assays (ARA) were performed: tubes previously sealed with rubber stoppers were injected with 0.5 mL pure acetylene and incubated at 30 ± 2°C for 24 h; acetylene and ethylene were assayed by a Packard model 419 Becker gas chromatograph equipped with a flame ionization detector and 6f alumina column. Tubes exhibiting both microaerophilic growth and acetylene reduction were scored positive for the presence of *Azospirillum* sp. and counted by the most probable number (MPN) method using McGrady's probability tables (Postgate 1969; Okon et al. 1977).

Bacterial inoculation and determination in wheat roots

Three wheat plants (*Triticum aestivum* cv. Hazera-18) were planted in 3-L pots containing one of the following soil types: brown–red degrading sand-soil of Rehovot; Loess raw soil of Kibbutz Nir-am, northwestern Negev or alluvial soil of Kibbutz Negba, northern Negev. Bacteria were grown in NB medium for 24–48 h at 30 ± 2°C, harvested by centrifugation at 12000 × *g* for 10 min, and washed twice with sterile tap water. Following seedling emergence, each pot

[1]Recipient of a Sir Charles Clore fellowship.

TABLE 1. Media used in the course of developing a selective media for *Azospirillum brasilense* Cd

Medium description	Abbreviation	Reference
Nitrogen-free medium prepared with Noble agar (Difco) and containing malate as a carbon source	OAB	Okon et al. 1977
OAB semisolid medium prepared with 0.05% Noble agar	SS	Okon et al. 1977
SS medium + 200 mg/L streptomycin sulphate	SSS	
General rich medium	King B	King et al. 1954
King-B medium + streptomycin sulphate (200 mg/L)		
Nutrient broth (Difco)	NB	
NB + streptomycin sulphate (200 mg/L)	NBS	
Nitrogen-free medium + Congo red (4 g/L)	RC	Rodríguez Caćeres 1982
OAB as a basal medium supplemented with the following: Streptomycin sulphate (200 mg/L) Cycloheximide (250 mg/L) Sodium deoxycholate (200 mg/L) 2,3,5-triphenyltetrazolium chloride (15 mg/L)	BL	Current work
BL medium supplemented with an aqueous solution of Congo red (1 g/L)	BLCR	Current work

was inoculated with 30 mL bacterial suspension of 10^7 colony-forming units (CFU) per millilitre. Control plants were irrigated with tap water at inoculation time. The soil surface of each pot was covered with a layer of 2−3 cm sterile vermiculite to prevent dispersion of the bacteria during irrigation. Plants were grown in an airconditioned greenhouse at 22−25°C and sampled for enumeration of *A. brasilense* Cd at 0, 4, 14, and 45 days after inoculation; 10 to 12 inoculated and control plants from the different soils were sampled each time. Plant roots were washed with a gentle spray of tap water until visibly clean of soil particles. One gram of whole young roots from each pot was asceptically placed into SSS enrichment medium and incubated for 48−72 h at 30°C. A liquid sample (0.5 mL) from the turbid zone was serially diluted and transferred into a fresh SSS medium, incubated as above, and counted by the MPN method. Additionally, a liquid sample (0.1 mL) from the fresh SSS medium was serially diluted, plated with a glass rod on BL solid medium, and incubated 96−120 h at 30 ± 2°C. The BL plates were inspected for morphological characteristics of *A. brasilense* Cd colonies. This procedure comprised the improved selective technique.

Experimental design and statistical analysis

All *in vitro* experiments were conducted in triplicate, each composed of three Petri dishes or three tubes. Experiments involving plants were conducted in five replicates, each composed of five pots. Each experiment was repeated three to five times and the results given are from a representative experiment. Bacterial counts are means calculated from five replicates and the standard error for each mean is indicated.

Results and discussion

Effect of various inhibitory substances on the growth of A. brasilense *Cd*

The effect of each of the 21 substances added to the King-B medium on the growth of *A. brasilense* Cd is given in Table 2. Four levels of inhibition were distinguishable: total growth inhibition (chloramphenicol, methyl violet, tetracyclin, KCN at 120 mg/L, and $CaCl_2$); a moderate inhibitory effect, particularly at low levels of inoculation (nalidixic acid, methyl green + methyl violet, KCN up to 60 mg/L, NaN_3, $ZnCl_2$, $K_2Cr_2O_7$, $CuCO_4$, $Cu(OH)_2$, and $3Cu(OH)_2 \cdot CuCl_2$); a low inhibitory effect (penicillin G, tetrazolium chloride, neomycin sulphate, nitrofurantion, and KCN at low levels); and no apparent effect (cycloheximide, streptomycin sulphate, sodium deoxycholate acid, and 2,3,5-triphenyltetrazolium chloride). Thus, the latter four substances were selected for further studies.

Addition of the above substances to the OAB medium formed BL selective medium. The growth of a pure culture of *A. brasilense* Cd on this medium was decreased with a recovery of 25−35% of the original population (see example in Table 3, column 6). Reducing the substances concentrations to $\frac{1}{2}$, $\frac{1}{4}$, or $\frac{1}{8}$ of the initial level did not increase the bacterial recovery. However, at the lower concentrations other bacterial species obtained from wheat rhizosphere such as pure cultures of *Bacillus* sp., *Pseudomonas* sp., and the more common nitrogen-fixing bacteria *Azotobacter* sp., *Klebsiella* sp., and *Erwinia herbicola* developed too (Buchanan and Gibbons 1974; Rennie 1981).

Five to eight days following incubation on the BL medium at 30 ± 2°C, light pink and colorless colonies were observed, which became gradually either dark pink or with a dark pink center and opaque colorless edges. When transferred into King-B medium the bacteria developed the typical colony morphology of the Cd strain, i.e., dark pink with dry, protruding ridges. Phase-contrast microscopy of a wet drop, from a colony suspected of being Cd, revealed rods resembling *Azospirillum* cells. Incubation in SS medium for 48 h at 30°C produced a white, dense, undulating diffused pellicle, 0.5 to 1.5 cm below the surface. The colonies had a positive ARA test, indicating N_2-fixing capability. Diagnostic biochemical and physiological tests were conducted by the methods described by Tarrand

TABLE 2. Effect of various substances added to King B medium on colony-forming units of *A. brasilense* Cd plated at 10^4 and 10^9 CFU levels

Inhibition type	Concentration (mg/L)	CFU of bacteria plated	CFU of bacteria detected
Control			
None		10^9	$2.1 \pm 0.2 \times 10^9$
None		10^4	$11.1 \pm 0.22 \times 10^4$
Total inhibition			
Chloramphenicol	250	10^9	0
		10^4	0
Tetracyclin	200	10^9	0
		10^4	0
KCN	120	10^9	0
		10^4	0
$CaCl_2$	10	10^9	0
		10^4	0
Methyl violet	60	10^9	0
		10^4	0
Moderate inhibition			
Nalidixic acid	33	10^9	$4.1 \pm 1.2 \times 10^4$
		10^4	0
Methyl green + methyl violet	60	10^9	$8.4 \pm 1.4 \times 10^1$
		10^4	0
KCN	20	10^9	$8.4 \pm 1.7 \times 10^5$
		10^4	$4.6 \pm 0.9 \times 10^1$
	30	10^9	$7.1 \pm 2.6 \times 10^5$
		10^4	$6.6 \pm 1.8 \times 10^1$
	60	10^9	$4.1 \pm 2.1 \times 10^3$
		10^4	0
NaN_3	10	10^9	$9.6 \pm 0.8 \times 10^4$
		10^4	0
	100–1000	10^9	0
		10^4	0
$ZnCl_2$	10	10^9	$4.7 \pm 2.7 \times 10^3$
		10^4	0
$K_2Cr_2O_7$	10	10^9	$6.1 \pm 0.9 \times 10^3$
		10^4	0
$CuSO_4$	10	10^9	$4.7 \pm 0.6 \times 10^2$
		10^4	0
$Cu(OH)_2$	15	10^9	$4.9 \pm 1.5 \times 10^2$
		10^4	0
$3Cu(OH)_2 \cdot CuCl_2$	15	10^9	$8.8 \pm 4.4 \times 10^3$
		10^4	0
Low inhibition			
Penicillin G	200 000 (units)	10^9	$7.1 \pm 2.1 \times 10^7$
		10^4	$3.6 \pm 1.7 \times 10^1$
Tetrazolium chloride	30	10^9	$3.0 \pm 0.6 \times 10^8$
		10^4	$1.7 \pm 0.4 \times 10^2$
Neomycin sulphate	200	10^9	$1.8 \pm 0.2 \times 10^6$
		10^4	$8.4 \pm 1.6 \times 10^2$
Nitrofurantion	10	10^9	$6.7 \pm 1.1 \times 10^6$
		10^4	$4.8 \pm 1.9 \times 10^2$
KCN	5	10^9	$8.8 \pm 1.6 \times 10^6$
		10^4	$6.6 \pm 1.4 \times 10^2$
	10	10^9	$3.6 \pm 1.1 \times 10^6$
		10^4	$3.1 \pm 0.9 \times 10^2$
No inhibition			
Cycloheximide	250	10^9	$9.6 \pm 1.4 \times 10^8$
		10^4	$7.4 \pm 1.8 \times 10^3$
Streptomycin sulphate	200	10^9	$1.8 \pm 0.2 \times 10^9$
		10^4	$0.9 \pm 0.1 \times 10^4$
Sodium deoxycholate	200	10^9	$1.0 \pm 0.4 \times 10^9$
		10^4	$8.6 \pm 0.6 \times 10^3$
2,3,5-Triphenyltetrazolium chloride	15	10^9	$1.6 \pm 0.3 \times 10^9$
		10^4	$0.7 \pm 0.1 \times 10^4$

TABLE 3. Detection of *A. brasilense* Cd on various diagnostic and selective media

Medium	CFU of *A. brasilense* detected 4 days after inoculation[a]	No. of different bacterial colonies	Total bacterial counts from wheat per gram roots	CFU of *A. brasilense* out of 10^9 CFU/mL plated[b]
General rich medium (King-B)[c]	ND	50–60	$4.7\pm0.6\times10^7$	$4.8\pm1.6\times10^9$
King-B + streptomycin sulphate	ND	15–20	$8.7\pm0.4\times10^4$	$8.9\pm0.7\times10^8$
Nitrogen-free medium (OAB)	$6.4\pm2.1\times10^2$	25–30	$3.6\pm0.7\times10^5$	$3.7\pm1.9\times10^9$
Nitrogen-free medium + Congo red (RC)	$8.7\pm0.8\times10^2$	10–15	$7.6\pm1.4\times10^5$	$4.1\pm0.9\times10^8$
RC + streptomycin sulphate	$6.7\pm1.1\times10^2$	10–15	$3.8\pm1.6\times10^5$	$4.7\pm1.1\times10^8$
OAB medium + 4 inhibitory substances (BL)	$2.6\pm0.7\times10^5$	5–7	$3.2\pm0.3\times10^5$	$7.6\pm0.2\times10^8$
BL medium + Congo red	$1.4\pm0.4\times10^5$	2–3	$1.8\pm0.6\times10^5$	$4.4\pm0.6\times10^8$

[a]ND, not detected.
[b]1.05 absorbance units at 540 nm.
[c]Control counts.

TABLE 4. Effect of Congo red addition to the OAB and BL selective media on growth of *A. brasilense* Cd

	Congo red concentration (g/L)			
Medium	0	1	2	4
OAB	$3.32\pm0.1\times10^9$*	$1.97\pm0.17\times10^9$	$1.58\pm0.17\times10^9$	$1.49\pm0.11\times10^9$
BL	$8.8\pm0.14\times10^8$	$7.5\pm0.17\times10^8$	$4.7\pm0.08\times10^8$	$4.45\pm0.13\times10^8$

*Values are given as colony-forming units.

TABLE 5. Effect of various liquid enrichment media on the detection of *A. brasilense* Cd in wheat roots, grown in brown–red degrading sand soil of Rehovot, 5 days after inoculation

Enrichment medium	CFU of *A. brasilense* Cd determined by the MPN method	CFU of *A. brasilense* Cd developed after enrichment and detected by the dilution method[a]	CFU of total bacteria developed in enrichment media and detected by the dilution method[b]	CFU of bacterial types developed in enrichment media
OAB semisolid medium (SS)	$3.4\pm0.6\times10^2$	$4.7\pm1.6\times10^6$	$5.8\pm0.7\times10^9$	30±5
SS medium + streptomycin (SSS)	$1.6\pm0.3\times10^6$	$6.1\pm1.1\times10^9$	$7.3\pm0.4\times10^9$	5±2
Nutrient broth (NB)	ND[c]	ND	$1.1\pm0.2\times10^{10}$	54±8
NB + streptomycin (NBS)	ND	$3.3\pm0.7\times10^3$[d]	$6.1\pm0.8\times10^9$	26±6

[a]On King-B + streptomycin.
[b]On King-B medium.
[c]ND, not determined.
[d]Counted on plates containing 200–400 colonies/plate.

et al. (1978). Identification of *A. brasilense* Cd from BL or SSS media was also carried out by the highly specific enzyme-linked immunosorbent assay, recently developed (H. Levanony, Y. Bashan, and Z. E. Kahana, unpublished data). All these tests confirmed the identification of the strain.

Addition of 4 g/L of Congo red to the autoclaved BL medium to produce the BLCR medium, did not reduce the number of *A. brasilense* Cd developed on the medium (Table 4). However, the colonies had a different appearance: they were relatively smaller (0.5–1 mm in diameter), round and dark red, apparently absorbing the red strain from their surroundings to produce a colorless plaque on the medium around each colony. The growth rate was slower than on the BL medium, and 2–3 additional days of incubation were required. However, fewer types of bacteria isolated from wheat roots developed on the BLCR medium, indicating its higher selectivity (Table 3, column 2). The disadvantage of the BLCR medium was the different appearance of the colonies, thus requiring additional transfer and growth on King-B medium.

Reducing the concentration of Congo red to 1 g/L, either in the BL or the OAB medium, resulted in a slight increase in the detected number of bacteria (Table 4). Thus, the lower level of Congo red was used for further studies. Another advantage of addition of Congo red to the medium was the total control of the fungal growth obtained from root extraction samples.

Enumeration of bacteria in several semisolid enrichment media

Four semisolid media were tested for counting *A. brasilense* Cd by the MPN method. However, media based on nutrient broth (NB and NBS) were not selective for *A. brasilense* Cd. Moreover, bacteria grown on these media failed to exhibit N_2-fixing capability. SS medium was also nonselective, allowing the development of many different bacterial strains. Enumeration was better on the more selective SSS medium on

TABLE 6. Growth of different strains of *Azospirillum* and other rhizosphere bacteria on BL and BLCR media

Bacterial strains	CFU of bacteria[a]		
	BL	BLCR	King-B[b]
A. brasilense			
Cd	$7.1\pm0.9\times10^8$	$3.1\pm1.2\times10^8$	$4.6\pm1.4\times10^9$
Cdw-1	$8.1\pm1.4\times10^8$	$4.1\pm0.8\times10^8$	$7.7\pm0.6\times10^9$
Cdw-3	$7.9\pm1.7\times10^8$	$2.8\pm0.7\times10^8$	$6.1\pm1.8\times10^9$
FT-400	$6.8\pm0.7\times10^8$	$4.1\pm0.3\times10^8$	$5.1\pm1.1\times10^9$
FT-326	$4.6\pm0.9\times10^8$	$3.2\pm1.2\times10^8$	$5.8\pm1.8\times10^9$
FT-339	$5.3\pm1.9\times10^8$	$3.3\pm1.1\times10^8$	$1.4\pm1.2\times10^9$
Azospirillum			
T_1-82008	$9.6\pm1.4\times10^8$	$1.2\pm0.7\times10^8$	$8.8\pm0.7\times10^9$
T_2-82012	$9.3\pm1.9\times10^8$	$0.8\pm0.3\times10^8$	$9.6\pm1.4\times10^9$
82009	$7.1\pm1.1\times10^7$	$6.6\pm1.3\times10^7$	$1.1\pm0.1\times10^{10}$
82013	$7.7\pm1.2\times10^7$	$4.1\pm1.6\times10^7$	$1.4\pm0.6\times10^{10}$
Pseudomonas			
82006	$4.4\pm1.7\times10^6$	$8.8\pm2.1\times10^5$	$8.9\pm1.8\times10^{10}$
82011	0	0	$2.4\pm0.8\times10^{10}$
82021	0	0	$7.1\pm1.1\times10^{10}$
Unidentified N_2 fixing			
780	$8.7\pm1.7\times10^8$	$6.1\pm1.6\times10^7$	$6.7\pm1.8\times10^{10}$
760	$4.3\pm0.7\times10^8$	$5.7\pm1.8\times10^7$	$9.9\pm1.4\times10^{10}$

[a] 1.05 absorbance units at 540 nm.
[b] Control counts.

TABLE 7. Isolation of *A. brasilense* from inoculated wheat plants cv. Hazera-18 grown in brown–red degrading sand soil of Rehovot by the selective method

Time after inoculation (d)	CFU of *A. brasilense*/ g roots in dilution count on BL medium	CFU of *A. brasilense*/ g roots by the MPN method	Total count of CFU/g roots on King-B medium	No. of different types of bacteria on BL medium	CFU of *A. brasilense*/ g roots by the selective method	Total count of bacteria/g roots by enrichment in SSS medium and counts on BL medium	No. of different types of bacteria grown by the selective method
0	$2.3\pm0.6\times10^5$	$1.8\pm1.6\times10^6$	$1.6\pm0.6\times10^7$	6	$4.4\pm1.7\times10^4$	$3.1\pm1.7\times10^7$	7
4	$4.6\pm0.7\times10^4$	$1.3\pm0.8\times10^6$	$8.1\pm1.4\times10^6$	5	$8.7\pm1.1\times10^4$	$4.7\pm1.2\times10^5$	5
14	ND[a]	$4.7\pm0.9\times10^5$	$6.6\pm0.8\times10^6$	5	$6.1\pm2.1\times10^3$	$6.6\pm0.9\times10^4$	5
45	ND	$8.9\pm1.7\times10^4$	$7.1\pm1.1\times10^6$	5	$3.6\pm0.8\times10^3$	$7.1\pm1.4\times10^4$	5

[a] ND, not detected.

which only a relatively small number of other types of bacteria developed. Also, *A. brasilense* Cd comprised most of the total bacteria developed on this medium while on the other media its growth was significantly lower. Thus, SSS medium was selected for enumeration of *A. brasilense* Cd by the MPN method (Table 5).

Comparison of several diagnostic and selective media for growth of A. brasilense *Cd*

Direct counts of wheat root microflora on a rich medium revealed more than 10^7 CFU/mL and more than 50 types of colony morphology. Generally in progressively more selective media, there was a decrease in diversity of bacterial types, in total counts and a significant increase in the number of detected *A. brasilense* Cd. The maximal selectivity was revealed on the BL medium, on which most of the bacteria were *A. brasilense* Cd. The minimal number of interference with other bacteria was detected on BLCR medium (2–3 different types of colonies as compared with 25–30 on the OAB medium) (Table 3).

Growth of different strains of Azospirillum *on BL and BLCR media*

In addition to *A. brasilense* Cd, other strains of *Azospirillum* and rhizosphere bacteria were tested for their ability to grow on BL and BLCR media. Out of 15 strains tested (see Materials and methods), all *Azospirillum* spp. and two other rhizosphere bacteria grew on BL and on BLCR medium (Table 6).

Isolation of A. brasilense *Cd from inoculated wheat plants by the selective technique*

Inoculated wheat plants were tested for the presence of *A. brasilense* Cd, at 0, 4, 14, and 45 days after inoculation (Table 7). Direct isolation of the bacteria, after root homogenization, spread of serial dilutions on agar medium, and counting the developing colonies was possible only 4 days after inoculation. MPN counts on SSS medium revealed a moderate decrease in *A. brasilense* Cd population with time. Total bacterial counts during the whole experiment were constant at around 10^6-10^7 CFU/g root and also the diversity of bacterial types developed on the BL medium remained constant. The

count of *A. brasilense* by the selective technique was one to two levels of magnitude higher than that of five to seven other types of bacteria. More than 10^3 *A. brasilense* Cd CFU/g root were detected and identified 45 days after inoculation. Soil type had no effect on recovery of *A. brasilense* Cd from wheat roots. However, separation of the roots from light-texture soil particles was easier as compared with the heavy soils.

Conclusions

The improved selection technique for the detection and enumeration of *A. brasilense* presented in this study was found to be superior to other techniques tested. The technique requires the following steps: placing washed roots into SSS medium followed by a transfer of a bacterial sample into a new SSS medium for evaluating ARA and microaerophilic growth, MPN determination, and isolation on BL medium based on colony morphology. The technique required minimal skill, allows for a simultaneous treatment of many samples, and is recommended for routine field inspection. However, since it does not provide an absolute identification of *A. brasilense*, a more specific technique, such as an immunological method, is needed. Additionally, the method has a disadvantage by the slow growth rate of the bacteria on the selective media.

Acknowledgements

This paper was written in memory of the late Mr. Avner Bashan for his constant encouragement and interest during this research. We thank Mrs. Adi Ravid Ben-Yehuda, Miss Orna Zamir, and Miss Orit Ziv for their excellent technical assistance, and Mr. Y. Avivi for very careful editing of the manuscript during its preparation. Overproducing auxin mutants of *A. brasilense* were kindly supplied by Dr. A. Hartman and Dr. M. Singh from Bayreuth University, Federal Republic of Germany.

BALANDREAU, J. 1983. Microbiology of the association. Can. J. Microbiol. **29**: 851–859.

BUCHANAN, R. E., and N. R. GIBBONS. 1974. Bergey's manual of determinative bacteriology. 8th ed. Williams and Wilkins, Baltimore, MD.

DE-POLLI, H., B. B. BOHLOOL, and J. DÖBEREINER. 1980. Serological differentiation of *Azospirillum* species belonging to different host-plant specificity groups. Arch. Microbiol. **126**: 217–222.

DÖBEREINER, J., and J. M. DAY. 1974. Associative symbiosis in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites. *In* Proceedings of the 1st International Symposium on Nitrogen Fixation. Vol. 2. *Edited by* W. E. Newton and C. J. Nyman. Washington State University Press, Pullmann. pp. 518–538.

KAPULNIK, Y., and Y. OKON. 1983. Benefits of *Azospirillum* inoculation on wheat: effect on root development, mineral uptake, nitrogen fixation and crop yield. *In Azospirillum* II, experientia supplementium. Vol. **48**. *Edited by* W. Klingmuller. Birkhauser Verlag, Basel, Boston, Stuttgart. pp. 163–170.

KING, E. D., M. K. WARD, and D. E. RANEY. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44**: 301–307.

MILLET, E., and M. FELDMAN. 1984. Yield response of a common spring wheat cultivar to inoculation with *Azospirillum brasilense* at various levels of nitrogen fertilization. Plant Soil, **80**: 255–259.

OKON, Y., S. L. ALBRECHT, and R. H. BURRIS. 1977. Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Appl. Environ. Microbiol. **33**: 85–88.

POSTGATE, J. R. 1969. Viable counts and viability. *In* Methods in microbiology. Vol. 1. *Edited by* J. R. Norris and D. W. Ribbons. Academic Press, London. pp. 611–628.

RENNIE, R. J. 1980. Dinitrogen-fixing bacteria: computer-assisted identification of soil isolates. Can. J. Microbiol. **26**: 1275–1283.

RENNIE, R. J. 1981. A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. **27**: 8–14.

REYNDERS, J., and K. VLASSAK. 1982. Use of *Azospirillum brasilense* as biofertilizer in intensive wheat cropping. Plant Soil, **66**: 217–223.

RODRÍGUEZ CAĆERES, E. A. 1982. Improved medium for isolation of *Azospirillum* spp. Appl. Environ. Microbiol. **44**: 990–991.

SCHANK, S. C., R. L. SMITH, G. C. WEISER, D. A. ZUBERER, J. H. BOUTON, K. H. QUESENBERRY, M. E. TYLER, J. R. MILAM, and R. C. LITTELL. 1979. Fluorescent antibody technique to identify *Azospirillum brasilense* associated with roots of grasses. Soil Biol. Biochem. **11**: 287–295.

TARRAND, J. J., N. R. KRIEG, and J. DÖBEREINER. 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can. J. Microbiol. **24**: 967–980.