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Enzyme-Linked Immunosorbent Assay for Specific Identification and Enumeration of *Azospirillum brasilense* Cd. in Cereal Roots†

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The enzyme-linked immunosorbent assay is suggested as a reliable, sensitive, and highly specific method for the identification and enumeration of *Azospirillum brasilense* Cd. As few as 10^5 CFU/ml can be practically identified by this method. At higher bacterial numbers, sensitivity increased linearly up to 5×10^8 CFU/ml, yielding useful standard curves. No cross-reaction was found either with different closely related *Azospirillum* strains or with other rhizosphere bacteria. The method allows for a specific identification of *A. brasilense* Cd. both in pure cultures and in mixtures with other bacterial species, even when the colony morphology is variable. The method was successfully applied to assess the degree of root colonization on various cereals by *A. brasilense* Cd.

After plants are inoculated with a specific bacterial strain, it is essential to use a reliable method for identification and enumeration of the applied strain, both for monitoring the applied bacteria and for evaluating the efficiency of the inoculation process itself. This task is particularly difficult because soil and rhizosphere bacteria are more variable than are their close relatives in other habitats. Moreover, many similar strains are likely to occupy and exploit the same microhabitat. In fact, related isolates, which differ from each other by one or a few characteristics, tend to compose a continuous spectrum, linked by a multitude of intermediate strains. In addition, the soil and the rhizosphere support enormous bacterial populations; thus, any attempt to detect a specific strain by using selective media was usually unsuccessful (14).

Bacteria of the genus *Azospirillum* are used for plant inoculation mainly because of their potential contribution to the yield of various cereals (3, 21, 24, 27). Two approaches were previously proposed for the identification and enumeration of *Azospirillum* strains, although both had limited success. In the first approach, several selective media (1, 4, 12, 25, 26, 28) were proposed, but they all allowed the development of many *Azospirillum* strains. The second approach, the fluorescent antibody technique (11, 30), was of a limited value for quantitative studies, although it was useful for the identification of a particular isolate. This technique suffered from additional drawbacks, including autofluorescence of the stelar portion of the root, some nonspecific binding of the fluorescent conjugate, and the need to scan many microscopic fields of each sample for statistical analysis.

During the last decade, the enzyme-linked immunosorbent assay (ELISA) was introduced for the specific identification of microorganisms. The method was adopted mainly by virologists for the detection of various viruses in plant (7) and animal tissues. In plants, however, application of the ELISA method has been less successful, being limited to symbiotic rhizobia (5, 16, 17, 19, 20, 22, 23). Several experiments on the detection of bacteria in plant tissues by the ELISA method failed to achieve the desired results, and these studies were probably discontinued (6, 9, 31; L. E. Claflin and J. K. Uyemoto, Phytopathol. News, p. 156, 1978).

The aim of the present study was to develop an ELISA technique for the specific identification and quantification of *A. brasilense* Cd. in pure cultures, in bacterial mixtures, and in the rhizosphere of various inoculated cereals.

MATERIALS AND METHODS

Bacteria. *Azospirillum brasilense* Cd. (ATCC 29710) was used as our standard strain. Other bacteria used were *A. brasilense* Sp-7 (ATCC 29145); auxin-overproducing mutants of *A. brasilense*, FT-326 and FT-400 (15), kindly provided by A. Hartmann and M. Singh from Bayreuth University, Bayreuth, Federal Republic of Germany; the *A. brasilense*-like strains 82008 and 82012 (2); rhizosphere bacteria strains 84072, 82013, and 82021, isolated from wild relatives of wheat growing in Israel; other rhizosphere bacteria, strains 1013, 1015, 1019, 1020, and 1023, isolated from roots of cultivated wheat; and the saprophytic strain 82005, were from our laboratory collection (Y. Bashan and H. Levanony, unpublished data); and the pepper leaf pathogen *Xanthomonas campestris* pv. *vesicatoria* (ATCC 11633).

Materials. The following materials were used: nutrient broth and complete Freund adjuvant (Difco Laboratories, Detroit, Mich.), DEAE-cellulose (Whatman Ltd., Kent, England), egg albumin, bovine serum albumin, and polyvinylpyrrolidone (PVP 10; Sigma Chemical Co., St. Louis, Mo.), goat anti-rabbit immunoglobulin G (IgG) coupled to alkaline phosphatase (A_{400} of 1.25 for a 1:3,000 dilution after 30 min of incubation at room temperature) (Miles-Yeda, Kiryat Weizmann, Rehovot, Israel), disodium paranitrophenylphosphate and Tween-20 (Sigma), and diethanolamine (E. Merck AG, Darmstadt, Federal Republic of Germany). The following microtiter plates were used: ELISA immunoassay plates (Linbro-Titertek 76-381-04; Flow Laboratories, Irvine, Scotland), immunoplate II 96F (Nunc, Roskilde, Denmark), and Microelisa plate M129A (Dynatech, Denkendorf, Federal Republic of Germany). The tissue culture plates used were Titertek 76-002-05 (Flow Laboratories), cluster 3569 (Costar, Cambridge, Mass.), and Nunclon (Nunc).

Antisera production. Whole cells of *A. brasilense* Cd. were used to elicit antibodies. Bacteria were grown in nutrient

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broth for 48 h at 30 ± 2°C in a rotary shaker (200 rpm). Cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4 ± 1°C and washed three times in sterile phosphate-buffered saline (PBS), pH 7.2, and their number was adjusted to 10^9 CFU/ml (1.05 A_{540} units). The bacterial suspension was emulsified with an equal volume of complete Freund adjuvant. Antibodies were elicited in New Zealand White rabbits by immunization with multiple intradermal injections with 1 ml of bacterial emulsion at four 1-week intervals, and a booster was given after two more weeks. Bleeding via cardiac puncture was started in week 2 postimmunization and continued for 3 months at 10-day intervals. Antisera from individual bleedings were stored at −20°C. Before use, the antisera were tested for their ability to induce agglutination, by using 10^8 CFUs of the antigen suspended in 200 μl of PBS in microtiter plates. The antisera used in this work had an initial titer of 1:512 by this method.

IgG purification. To minimize nonspecific interactions, γ-globulin was partially purified as follows. Samples (1 ml) of antisera were diluted 10-fold with distilled water, and 10 ml of a saturated solution of ammonium sulfate (pH 5.5) was then added to each diluted sample. After 1 h at room temperature, the formed precipitate was collected by centrifugation at 12,000 × *g* for 15 min, dissolved in 2 ml of half-strength PBS, and dialyzed overnight at 4 ± 1°C against three changes of 500 ml of half-strength PBS–0.02% sodium azide. The IgG was further purified on a column (1 by 8 cm) of DEAE-cellulose (DE-23) (Whatman), which was previously equilibrated with half-strength PBS–0.02% sodium azide. The unadsorbed fraction was adjusted to 1 mg/ml (E_{280} = 1.4) and stored frozen at −20°C in 1-ml microtubes (Eppendorf Geratebau, Netheler, and Hinz GmbH, Hamburg, Federal Republic of Germany).

ELISA procedure. Two ELISA procedures were carried out: indirect ELISA (18) and competition ELISA (7).

(i) **Indirect ELISA.** Wells of microtiter plates were coated with the antigen as follows. Freshly prepared or thawed stored bacteria at 10^8 CFU/ml were suspended in a coating buffer (0.05 M sodium carbonate [pH 9.6] containing 0.02% sodium azide), and 200 μl of this suspension was allotted to each well. A similar coating procedure was followed for roots and for root extracts (see below). After an overnight incubation at 4 ± 1°C, the wells were washed three times at 3-min intervals with a washing buffer which consisted of PBS containing 0.05% Tween 20. Subsequently, the washing buffer was supplemented with 1% egg albumin and added to the wells to block unreacted sites. After 1 h at 37 ± 1°C, the wells were washed as described above. Wells were then filled with 200 μl of anti-*A. brasilense* Cd. IgG diluted 1:1,024 in PBS containing 0.05% Tween 20 and 0.02% sodium azide and incubated for 90 min at 37 ± 1°C. After all unbound antibodies were washed off, 200 μl of goat anti-rabbit IgG coupled to alkaline phosphatase was applied; the IgG conjugate was previously diluted 1:5,000 in PBS containing 0.05% Tween 20, 0.02% sodium azide, 2% polyvinylpyrrolidone, and 0.2% bovine serum albumin. Plates were incubated at 4 ± 1°C overnight. After three final washings, the plates were treated with a freshly prepared substrate solution of disodium paranitrophenyl phosphate dissolved at 0.1 mg/ml in 10% diethanolamine buffer (adjusted to pH 9.8) containing 0.05% sodium azide. The plates were then incubated at 37 ± 1°C for periods ranging from 2 to 24 h. The enzymatic reaction was recorded as the A_{405} with a Titertek Multiskan Photometer (Flow Laboratories).

(ii) **Competition ELISA.** Similarly to the indirect ELISA, microplates were coated with 5 × 10^7 CFU of bacteria per ml, and unbound antigens were similarly washed off. The specific antibodies elicited against *A. brasilense* Cd. were mixed with competitors (listed below), and the mixture was incubated for 90 min at 37 ± 1°C in glass test tubes. A 200-μl volume of the suspension was added to each well. The rest of the procedure, including incubation, washings, addition of goat anti-rabbit IgG conjugate, and color development was as described above.

The following rhizosphere bacteria were used as competitors: *A. brasilense* Cd. and Sp-7; the *A. brasilense*-like strains 82008 and 82012; the rhizosphere bacteria 1013, 1020, and 1023; the phytopathogenic bacteria *X. campestris* pv. *vesicatoria*; and the saprophytic bacteria strain 82005.

In all ELISA experiments, dilutions of 10^5, 10^6, and 10^7 CFU of *A. brasilense* Cd. per ml were incorporated in each plate as standards. Wells lacking the antigen but containing all other ELISA components served as blanks. Only the central part of each microtiter plate was used to minimize variation caused by the drying of outer rows during the incubation periods. A row of empty wells was routinely left between adjacent treatments to avoid mixing artifacts. All plates were covered and sealed with Parafilm and then wrapped in polyethylene bags. The microplates were incubated in a single layer.

Attempts were made in a few experiments to enhance the adsorption of the bacteria to the polystyrene plates by precoating the plates, before the application of the antigen, with one of the following: wheat germ agglutinin, 1 mg/ml; soybean agglutinin, 1 mg/ml; glutaraldehyde, 0.125 and 0.250%; concanavalin A, 0.5 and 1 mg/ml; protein A, 0.5 and 1 mg/ml; and poly-L-lysine, 1, 2, and 5 mg/ml (donated by H. Lis and Z. Eshchar, Weizmann Institute of Science, Rehovot, Israel). The enhancement of bacterial adsorption was also attempted by drying precoated plates with antigen in an air-ventilated oven at 45 ± 2°C for 24 h.

Inoculation of cereals with *A. brasilense* Cd. Several cereals were grown in glass dishes at 22 ± 2°C in a growth chamber (model EF7H; Conviron, Controlled Environments, London, England) and in pots at 22 ± 3°C in an air-conditioned greenhouse. These included common wheat (*Triticum aestivum* L. cv. Deganit), corn (*Zea mays* L. cv. jubilee), cultivated barley (*Hordeum sativum* Jess.), wild barley (*H. spontaneum* Koch), *Triticale* (amphiploid, wheat/rye), sorghum (*Sorghum bicolor* cv. 610), setaria (*Setaria italica* L.), and the hybrid sorghum × Sudan grass. Rye (*Secale cereale* L.) was grown only in the greenhouse. (Seeds of the summer cereals were kindly provided by Y. Okon, Hebrew University of Jerusalem, Rehovot, Israel, and seeds of winter cereals were provided by M. Feldman and E. Millet from our department).

Seeds were disinfected in 1% NaOCl for 2 min, rinsed thoroughly in tap water, allowed to imbibe for 3 to 4 h, and then transferred to glass dishes or to soil. Seeds grown in glass dishes were placed on wet filter paper (Whatman no. 1), and the seedlings were inoculated at 3 to 6 days with 2 ml of 10^9 CFU of *A. brasilense* Cd. per ml (2). Roots were cut and assayed for the presence of bacteria 24 to 96 h after inoculation. Alternatively, three seeds were sown per 1-liter pot containing brown-red degrading sand soil of Rehovot. After seedling emergence, each pot, excluding the controls, was inoculated with 10 ml of 10^9 CFU of *A. brasilense* Cd. per ml. Roots were tested by the ELISA technique at 4 to 14 days after inoculation.

Bacterial determination in roots. Bacteria from inoculated and control plants were prepared both from root sections and from root homogenates as follows. Roots, either slightly

washed or unwashed, were cut into pieces 3 to 5 mm long. These were immediately suspended in the coating buffer and placed into wells of microtiter plates. Concurrently, other root pieces were placed into nitrogen-free semisolid medium (4). At 48 to 72 h after incubation, bacterial bands that had developed in the semisolid medium about 1 cm below the surface were also used as an antigen source or as competitors in competition ELISA. Acetylene reduction in tubes that had shown bands was determined. The number of bacteria was calculated by the most probable number method as previously described (4).

Additionally, roots were homogenized by a disperser (Model × 10/20; Ystral, Ballrechten-Dottingen, Federal Republic of Germany) in 0.06 M potassium phosphate buffer, pH 7.0, in an ice bath. The slurry obtained was further homogenized by a fine glass homogenizer (Kontes, Vineland, N.J.). After centrifugation at 12,000 × *g* for 10 min, the pellet was dissolved in a minimum volume of 2 to 3 ml of buffer and used either for coating the wells or for competition ELISA. Concurrently, samples of homogenized roots were incubated in semisolid medium, and the bands obtained were also used for coating the wells or for competition ELISA. Serial dilutions of the root pellet suspension in 0.1-ml portions were plated with a glass rod on King-B medium and BL medium (4). All plates were incubated at 30 ± 2°C for 48 h, and the number of colonies was determined.

Bacterial counts from microtiter plates. After the bacterial coating, unadsorbed bacteria were pumped out aseptically, serially diluted in PBS, and counted by the plate count method on King-B medium 48 h after incubation at 30 ± 2°C (4).

Maceration of bacteria. Bacterial cultures were washed in PBS and serially diluted. Each bacterial dilution was macerated for 15 min at 140 W in an ice bath by using an ultrasonic disintegrator (Sonifier B-12; Branson Sonic Power Co., Danbury, Conn.).

Experimental design. All experiments were designed in a random fashion in triplicate, with five pots or two to six wells as a single replicate. Experiments were repeated 2 to 10 times each as indicated in the text. Each plate contained controls as indicated above, including a thawed culture of *A. brasilense* Cd. of a known dilution. This culture was used throughout the study for comparing different performances of the plates and various modifications in the ELISA technique itself during its development. Controls such as preimmune sera or wells with conjugate or substrate but without antibodies or antigens were also included.

RESULTS

Optimization of the ELISA conditions. In an attempt to find the optimal conditions for the ELISA, the following experimental variables were tested: antibody dilutions (from 1:1 to 1:1,024), dilutions of goat anti-rabbit enzyme conjugate (1:5,000 and 1:10,000), duration of incubation with the substrate (from 30 min to 24 h), and microtiter plates from various manufacturers and of different properties. All tests were done with a substrate concentration of 0.1 mg/ml.

Optimal results were obtained at antibody dilutions of 1:512 to 1:1,024 (i.e., 1 to 2 μg of IgG per ml) and at dilutions of goat anti-rabbit conjugate of 1:5,000 or 1:10,000. Under these conditions, the level of detected bacteria increased nearly linearly as the bacteria number increased; preimmune serum yielded negligible values (Fig. 1a). Extending the time of incubation with the substrate, either in the indirect (Fig. 1b) or in the competition (Fig. 1c) ELISA, yielded higher values, while other bacterial strains, as represented by strain 82012, were still undetected. On the basis of these findings, values were routinely measured 2 h after substrate incubation. In some cases, this incubation period yielded absorbance values that were too low, and the incubation was extended up to 24 h, yielding values of 1.1 to 1.5 absorbance units for 5×10^7 CFU/ml.

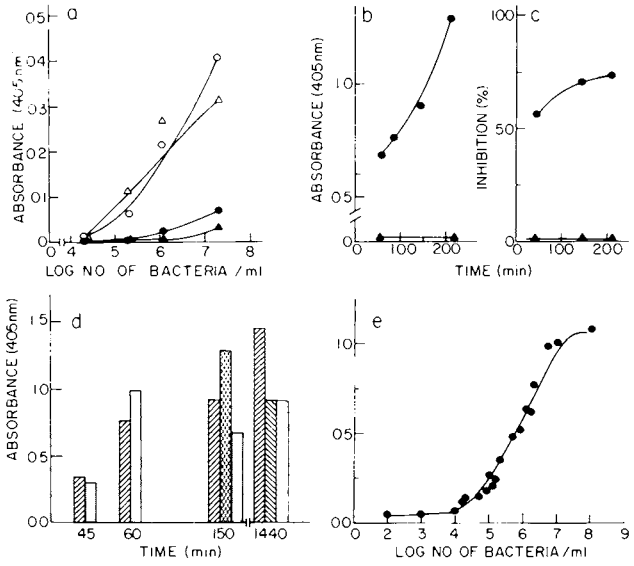

FIG. 1. Effect of various factors on the detection and enumeration of *A. brasilense* Cd. by the ELISA method. In all experiments, wells were coated with the bacteria overnight at 4 ± 1°C, incubation with antibody lasted 90 min, and incubation with the conjugate was overnight at 4 ± 1°C. (a) Detection of *A. brasilense* Cd. by preimmune serum at conjugate dilutions of 1:5,000 (●) and 1:10,000 (▲) and by immune serum at conjugate dilutions of 1:5,000 (○) and 1:10,000 (△). (b) Detection by indirect ELISA of *A. brasilense* Cd. (●) or of strain 82012 (▲) at different incubation periods with the substrate; in both cases, 5×10^8 CFU/ml was used. (c) Detection by competition ELISA of *A. brasilense* Cd. (●) and of strain 82005 (▲) at different incubation periods with the substrate; in both cases, 5×10^7 CFU/ml was used. (d) Detection of *A. brasilense* Cd. in various microtiter plates at different incubation periods with the substrate. In all cases, 5×10^7 CFU/ml was used. The plates used were Linbro/Titertek 76-381-04 (▨), Nunc immunoplate II 96F (▥), microelisa M129A (▤), tissue culture Titertek 76-002-05 (▩), tissue culture cluster 3596 (▧), and Nunclon (□). (e) Sensitivity curve of *A. brasilense* Cd. under the optimal experimental conditions of the indirect ELISA. Incubation time with the substrate was 115 min.

The type of microtiter plate used greatly affected the level of detection (Fig. 1d). Generally, Linbro enzyme immunoassay microtiter plates yielded the most satisfactory results, whereas tissue culture plates were excellent for detection of low levels of bacteria in plant material.

The optimal conditions of the indirect ELISA for the detection of *A. brasilense* Cd. can be summarized as follows: overnight antigen coating, antibody dilution of 1:1,024, conjugate dilution of 1:5,000, substrate concentration of 0.1 mg/ml, and incubation time of 2 h with the substrate for a pure culture and up to 24 h for low bacterial numbers in roots. Figure 1e shows a standard sensitivity curve of bacteria under these conditions. The technique detects, with high confidence and reproducibility, as few as 10^5 CFU of bacteria per ml.

The sensitivity of the ELISA was slightly affected by the

TABLE 1. Adsorption of *A. brasilense* Cd. by microtiter plates[a]

No. of applied bacteria[b]	No. of unadsorbed bacteria[c]	% Adsorption
7.5×10^3	2×10^2	97
7.5×10^4	1×10^3	98
7.5×10^5	1×10^4	98
7.5×10^6	3.7×10^5	95

[a] Means of three experiments.
[b] Bacteria were suspended in a coating buffer consisting of 0.05 M sodium carbonate at pH 9.6 and supplemented with 0.02% sodium azide; 0.15 ml was applied to each well in six replicates and incubated overnight at 4 ± 1°C.
[c] Unadsorbed bacteria were aseptically pumped out, serially diluted in PBS, and counted by the plate counting method on King-B medium after 48 h of incubation at 30 ± 2°C.

state of the antigen used. At a low number of bacteria, the values obtained for live, frozen or sonicated bacteria were similar; at a high number of bacteria, the values for frozen cultures were in most cases slightly higher than those for live bacteria. The possibility that alkaline phosphatase activity originated from *A. brasilense* Cd. under the conditions outlined was tested and excluded. Values obtained by using only coating buffer or solutions of blocking substances, but no antigen, antibody, or conjugate, were negligible (0 to 0.006 absorbance units).

Adherence of *A. brasilense* Cd. to microtiter plates. Only a small fraction (less than 5%) of the applied bacteria was not absorbed to the microplates (Table 1). To exclude death of the culture during the overnight incubation at 4 ± 1°C, cells of the same bacterial culture were stored in a 100-ml glass Erlenmeyer flask under the same conditions; no measurable bacterial death was revealed.

Blocking effects. Preliminary observations showed that after application of the bacteria, the uncoated areas of the plate should be masked; otherwise, the adsorption of antibodies to the unoccupied sites interfered in both ELISA procedures, especially in competition tests. The effectiveness of adding 1% egg albumin as a blocking agent was demonstrated (Fig. 2). Other blocking agents such as bovine serum albumin, ethanolamine, and bacterial cells of strains 1023 or *X. campestris* pv. *vesicatoria* were less effective, but

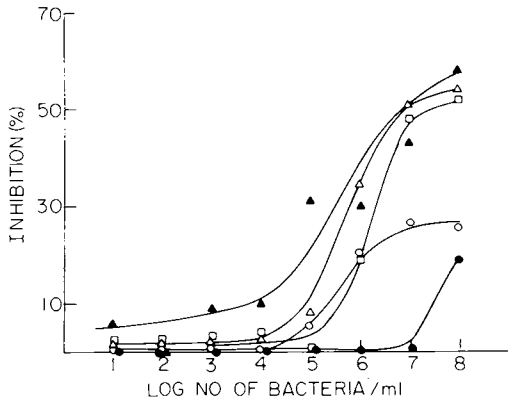

FIG. 2. Effects of different blocking treatments in competition ELISA. The treatments used were no blocking (●), 1% egg albumin (▲), 1% bovine serum albumin (○), 1% ethanolamine (□), and 10^9 CFU of isolate 1023 or *X. campestris* pv. *vesicatoria* per ml (△). Experimental conditions were as described for Fig. 1e. Data are presented without preincubation of antibody and bacteria.

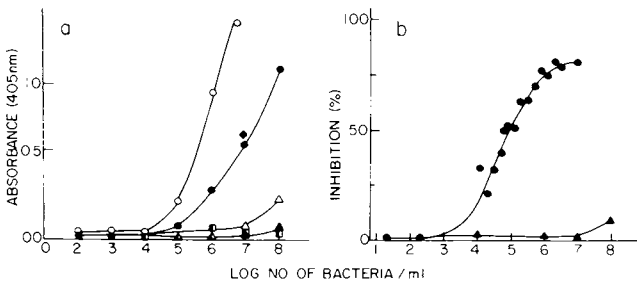

FIG. 3. (a) Specificity tests for the detection of various bacteria by the indirect ELISA method. The duration of substrate incubation is given in parentheses. Bacteria used were *A. brasilense* Cd. (60 min) (●), *A. brasilense* Cd. (220 min) (○), mutant FT-326 (60 min) (◆), strain 1019 (60 min) (▲), strain 1019 (220 min) (△), strain 1015 (60 min) (■), and strain 1015 (220 min) (□). (b) Competition ELISA at different concentrations of *A. brasilense* Cd. (●) and strain 82008 (▲). Plates were coated with 10^7 CFU of *A. brasilense* Cd. per ml overnight at 4 ± 1°C, blocking was done with 1% egg albumin for 60 min at 37 ± 1°C, preincubation of antibodies with competitors lasted for 90 min at 37 ± 1°C, incubation with the antigen lasted 90 min at 37 ± 1°C, incubation with the conjugate was overnight at 4 ± 1°C, and the enzymatic reaction took place at 37 ± 1°C for 120 min.

nevertheless decreased the interference caused by the unblocked sites.

Precoating treatments. Attempts to increase the sensitivity of the ELISA with several precoating substances (see Materials and Methods) were unsuccessful. Drying the plates that were coated with bacteria increased the values obtained but did not improve sensitivity. It increased the noise of the control wells, perhaps as a result of bacterial multiplication (1.68 absorbance units for 5×10^7 CFU/ml compared with 1.45 absorbance units without any pretreatment).

Specificity of ELISA. The specificity toward *A. brasilense* Cd. was tested both by indirect and by competition ELISA. The results obtained by the two methods were in full agreement. By the indirect ELISA, high binding values were obtained only for *A. brasilense* Cd., for its mutant FT-326 (Fig. 3a), for the mutant FT-400, and for one morphological mutant colony (0.8 ± 0.15 absorbance units) after both 1 and 3 h of substrate incubation. Negligible binding values (up to 0.05 absorbance units) were recorded for other rhizosphere bacteria including *A. brasilense* Sp-7, the *A. brasilense*-like strains 1015, 1019 (Fig. 3a), 82008, and 82012, and the rhizosphere bacteria 82013, 1020, and 1023, as well as for other strains including the phytopathogenic *X. campestris* pv. *vesicatoria* and the saprophytic 82005 strains (not shown). In all cases, such low values were obtained even when the tested bacteria were at very high numbers (10^{10} to 10^{11} CFU/ml). The sensitivity of the assay varied from 10^4 to 10^5 to 5×10^8 CFU/ml (Fig. 3a). Very similar results were obtained in the competition ELISA (Fig. 3b). When *A. brasilense* Cd. was used as a competitor, maximum inhibition of over 85% was obtained for 10^7 CFU/ml, and 50% inhibition was obtained for 8×10^4 CFU/ml. Other rhizosphere bacteria did not cause any inhibition under the testing conditions (Fig. 3b); only very high bacterial numbers (10^{10} to 10^{11} CFU/ml) caused some inhibition (<10%). Competition ELISA had a higher sensitivity than did the indirect method. Efficient blocking was essential for the performance of these tests, as was the process of preincubation of antibody with the competitor. Inhibition was diminished by low temperature and by insufficient incubation time.

Detection of enumeration of *A. brasilense* Cd. in wheat

TABLE 2. Enumeration of *A. brasilense* Cd. and the rhizosphere bacteria strain 1023 in inoculated wheat roots by various methods

Source of plant material	Coating antigen[a]	No. of *A. brasilense* Cd. (CFU/g of root)[b]			No. of strain 1023 cells (CFU/g of root)	
		By indirect ELISA	After enrichment; by indirect ELISA	By MPN[c]	By indirect ELISA	After enrichment
Filter paper	Root tips	10^5	ND[d]	ND	ND	ND
	Root extract	$\leq 10^4$	ND	2×10^4	ND	ND
Soil	Root tips	$<10^4$	2×10^4	ND	0	$0–10^2$
	Root extract	$<10^4$	5×10^4	6.6×10^3	ND	ND

[a] After washing.
[b] After subtraction of control values.
[c] MPN, Most probable number method as determined by Bashan and Levanony (4).
[d] ND, Not determined.

roots. When wells were coated with root tips or with root extracts of inoculated plants, *A. brasilense* Cd. could be identified by both ELISA methods. In both cases, root tips gave higher values than did root homogenates. Roots grown on filter paper yielded higher values than did those grown in the soil. Irrespective of the root source, bacteria in plants that were previously inoculated with *A. brasilense* Cd. could be quantified, while those inoculated with other rhizosphere bacteria (listed above) yielded negligible absorbance values. However, when very few *A. brasilense* Cd. cells ($<10^4$ CFU/g [fresh weight] of root) were present, the assay could be applied only after enrichment of the bacteria. In this case, bacterial enumeration should be done by the most probable number method (4) after enrichment of bacteria in semisolid medium; however, positive identification of the bacteria was performed by using the ELISA technique rather than by relying on the physiological characteristics of *A. brasilense* (Table 2).

Inoculation of wheat roots with other strains of rhizosphere bacteria, such as 1023 or 84072, or testing unidentified rhizosphere bacteria isolated at random from plants grown in uninoculated pots resulted in negligible values. The maximum inhibition value obtained by other rhizosphere bacteria was 12.5% of that obtained by *A. brasilense* Cd. The bacterial population present naturally on the root surface or the plant material itself did not interfere with the detection and enumeration of *A. brasilense* Cd.

Detection and enumeration of *A. brasilense* Cd. in roots of other cereals. *A. brasilense* Cd. could be detected and enumerated by both ELISA methods in the roots of the following nine different cereals grown in soil or on filter paper: cultivated wheat, cultivated barley, corn, triticale, sorghum, setaria, the hybrid sorghum × Sudan grass, rye, and wild barley (Fig. 4).

DISCUSSION

Serological techniques, such as fluorescent antibody and immunoprecipitation, are highly specific for identifying microorganisms (29). Recently, the advantages offered by the ELISA technique as a practical diagnostic method have been acknowledged. Its high sensitivity, which is similar to that of the radioimmunoassay techniques (13), and its applicability to quantitative studies (7) are noteworthy.

The level of specificity obtained in the identification of *A. brasilense* Cd. was similar to that reported for other immunological techniques (10, 11, 30). This specificity, which was most probably due to some exterior cell wall components, allows one to distinguish *A. brasilense* Cd. from closely related strains of the same species. The antiserum reacted specifically with the wild type and with its mutants. This specificity depended on the quality of the antiserum and not on the degree of its purification. Thus, only a partial purification of the antiserum was needed for the assay. There was almost no cross-reaction with other rhizosphere bacteria present on the roots or with plant material present in the extracts. Only very high numbers of these bacteria (10^{10} to 10^{11} CFU/ml), which normally do not exist naturally in the rhizosphere, gave limited cross-reactions. The reactions of dead or frozen *A. brasilense* Cd. were of the same magnitude as those of live bacteria. This fact enabled us to use the same *A. brasilense* Cd. culture as a standard control in every plate, which allowed comparison between experiments conducted over a prolonged period. These results are in accordance with those of Berger et al. (5), Cother and Vruggink (9), and Vruggink (31) and are in disagreement with the findings of Nambiar and Anjaiah (23), who detected only viable cells by the ELISA.

Unlike some other serological techniques, which are

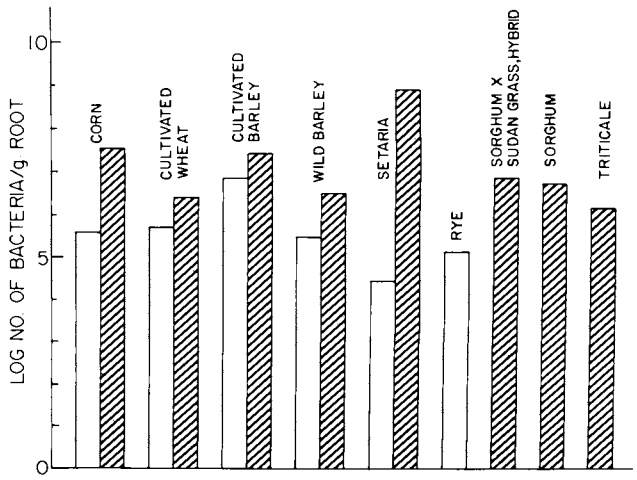

FIG. 4. Detection and enumeration of *A. brasilense* Cd. in roots of different cereals. Roots from an axenic culture, measured by indirect ELISA 3 days after inoculation (▨). Roots from soil, measured by competition ELISA 14 days after inoculation (□). Values obtained for uninoculated roots were subtracted from those of inoculated ones.

based on the formation and detection of an immune precipitate, the ELISA relies on the sensitive detection of binding reactions. Furthermore, the easily measurable intensity of the color produced is proportional to the amount of enzyme, which in turn is directly related to the quantity of the antigen. Thus, the ELISA is endowed with the specificity of antigen-antibody reaction and the sensitivity of enzyme-catalyzed reaction. This combination provides for sensitivity and specificity which are similar to those obtained by radioimmunoassay, without the inherent disadvantages of high cost, potential health hazard, and short shelf life of the radioactive reactants. Furthermore, in borderline cases, additional incubation time with the enzyme resulted in the development of detectable color, a procedure which could not be used in radioimmunoassay.

The major problem encountered when using the ELISA technique is the threshold number of bacteria detected. In general, differentiating noise from signals becomes progressively more difficult as the concentration of the antigen decreases. Kishinevsky and Bar-Joseph (16) detected 10^4 to 10^5 CFU of rhizobia per ml, Mårtensson et al. (20) detected 10^3 rhizobia per ml in only two *Rhizobium* strains, whereas Nambiar and Anjaiah (23) lowered the level to 10^2 to 10^3 rhizobia per ml. However, the data from the latter is about 0.1 absorbance units or less and is commonly agreed to be the background noise level (7). It is known that only rhizobia are present within the nodule, while the rhizosphere contains large numbers of different bacterial species. The sensitivity of the ELISA developed for *A. brasilense* Cd. was similar to the sensitivity reported by Kishinevsky and his co-workers working with rhizobia (16, 17). The lower limit was 10^4 to 10^5 cells per ml. Attempts to increase these values by pretreatment with various substances were unsatisfactory, because it was accompanied by an increase in noise. Competition ELISA tests confirmed the findings of indirect ELISA with slightly improved sensitivity. Therefore, the two ELISA versions are possible, but the more sensitive competition ELISA is recommended for root pieces or for root extracts of plants grown in soil, which usually yielded low values by the indirect method.

Despite the common use of the double antibody sandwich technique in ELISA procedures, i.e., application of the antibody to the plate, followed by the antigen and then conjugated antibody (7), we preferred to adopt the indirect ELISA version in which the antigen, namely, the bacteria, was adsorbed directly to the plate. This facilitated the enzymatic reaction with common goat anti-rabbit conjugate, which is commercially available, and precluded the need to separately produce such IgG for each antiserum. In addition, it enables the use of tissue culture plates especially designed for the adsorption of cells; these plates adsorb γ-globulins poorly (8).

A minor disadvantage of the ELISA technique is the variability encountered because of different plates. Thus, a known amount of *A. brasilense* Cd. was incorporated in every plate as a standard. In addition, caution should be exercised with root washings during the preparation of inoculated roots, since *A. brasilense* Cd. adheres rather loosely to wheat roots, and the number of bacteria might be relatively small (4a).

However, because of its versatility, precision, simplicity, and speed, the technique is suitable for large-scale testing of plant bacterium samples. This technique may be adopted to detect and monitor populations of other bacterial species, either beneficial or pathogenic, when applied to various plants.

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