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ESTIMATION OF MINIMAL NUMBERS OF *AZOSPIRILLUM BRASILENSE* USING TIME-LIMITED LIQUID ENRICHMENT COMBINED WITH ENZYME-LINKED IMMUNOSORBENT ASSAY

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Summary-A simple time-limited liquid enrichment procedure to aid in the quantitative detection of very few cells *of Azospirillum brasilense* in plant roots is described. The method is based on limited multiplication *of A. brasilense* in convential semi-solid medium and counting of the bacteria in the enriched medium by enzyme-linked immunosorbent assay (ELISA) or by Most Probable Number (MPN) techniques. The method can be used as a complementary procedure to ELISA and MPN techniques when low numbers of *A. brasilense* are present in the roots.

INTRODUCTION

Bacterial strains belonging to the genus *Azospirilum* are known for their positive effect on plant growth and productivity (Bashan and Levanony, 1990; Michiels *et al.*, 1989). The species *A. brasilense* together with *A. lipoferum* comprise the most studied species of *Azospirillum.* Efficient root colonization by cells of *Azospirillum* sp. after inoculation is essential to obtain a plant response to the presence of the bacteria. Enumeration of cells of *Azospirillum* sp. in the rhizosphere is a common control required in any study describing *Azospirillum*-plant interaction. Enumeration of its population is relatively simple when high numbers are present (10^5-10^7 cfu g^1 (fresh weight). Several methods based on the Most Probable Number (MPN) method combined with semi-selective media (Bashan and Levanony, 1985; Döbereiner *et al*., 1976; Rodriguez-Caceres, 1982), enzyme-linked immunosorbent assays (ELISA) (Levanony et al., 1987) and avidin-biotin complex incorporation to ELISA (Levanony and Bashan, 1990) have been used to satisfactorily evaluate populations of *Azospirillum* spp. However, when populations fall below the detection level of ELISA ($<10^4$ cfu/sample), or when other species of rhizosphere bacteria are present on the semi-selective media, quantitative detection of small numbers of an inoculated strain of *Azospirillum* spp is extremely difficult. Small numbers of *Azospirillum* spp in the rhizosphere have been detected frequently (Bashan *et al.*, 1987; Bashan and Wolowelsky, 1987; Harris *et al*., 1989; Nayak *et al*., 1986; Smith *et al.*, 1984).

To overcome the difficulty of detecting low numbers of *Azospirillum* spp, qualitative analysis employing liquid enrichment of root samples containing cells of *Azospirillum* spp in semi-selective media is frequently used. However, this method yields only positive or negative results regarding the presence of *Azospirillum* spp. The objective of the procedure described herein is quantitative detection of very low numbers of *A. brasilense* using time-limited (16 h) liquid enrichment combined with ELISA or plate count methods. This procedure is based on two assumptions. The first assumption is that *Azospirillum* multiplies in N-free semi-solid medium in a defined microaerophilic pellicle, which is a characteristic of the genus. The second assumption is when a small population of *A. brasilense* is present in a root sample, upon time-limited enrichment in N-free semi-solid medium, these bacteria will develop a smaller population during the logarithmic phase of growth in the predicted site for pellicle formation compared to a population developed from a larger initial *Azospirillum* spp population. However, the population which developed should be large enough to be deteced by conventional bacterial count methods.

MATERIALS AND METHODS

Organisms

The following *A. brasilense* strains were used: Cd (ATCC 29710), Sp-245 and Sp-246 (Baldani *et al.*, 1986) and Somali 67 isolated from mucilaginous cyanobacterial crust .collected from alluvial and ferruginous soils in Somalia, East Africa (Favilli et al., 1988). Spring wheat seedlings, *Triticum aestivum* cv. Deganit (Zeraim Gedera Co., Israel) were used as the model plants.

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Media and bacterial growth conditions

The following media were used: N-free semi-solid media (i) NFb (Döbereiner et al., 1976) for enrichment of *A. brasilense* Sp. 245 and Sp. 246. (ii) OAB (Bashan and Levanony, 1985) for enrichment of *A. brasilense* Somali-67; (iii) BL (Bashan and Levanony, 1985) for enrichment of *A. brasilense* Cd. All strains were enumerated primarily on nutrient agar (Difco). When cells of *A. brasilense* were accompanied by large numbers of other rhizosphere bacteria, enumeration was performed on the respective solid media (1.5% agar) listed above. The use of different semi-selective media for *A. brasilense* strains was aimed to give optimal growth conditions to each *Azospirillum* strain with minimal interaction with other rhizosphere strains. These media had been found to be optimal for these strains.

Time-limited enrichment technique

Bacteria analyzed by regression analysis were grown in nutrient broth (Difco) for 24 h (30 ± 1 °C, 250 rev min^{-1}), washed in 60 mM potassium phosphate buffer supplemented with 150 mM NaCl (PBS), diluted in the same buffer to the required inoculum dilution and 0.1 ml samples were inoculated into semi-solid media in test tubes. Inoculated tubes were stirred once by a vortex stirrer and grown at 30 ± 1°C without further movement of 16 h. Samples were taken from the predicted site of pellicle formation in the tube of a particular strain. The location of this site had been visually determined (in mm below solution surface) after growth of each strain for 3-5 days.

Populations of bacteria were identified and estimated by an indirect-ELISA method (Levanony *et al.*, 1987) for strain Cd and by the improved selection technique, based on MPN and the conventional plate count method on semi-selective medium, for the other strains (Bashan and Levanony, 1985). Different counting methods were used because specific antibodies are available only for *A. brasilense* Cd.

Plant growth conditions and inoculation

Wheat seedlings were grown in quartz sand in pots (300 ml) supplemented with half-strength Hoagland's nutrient solution for 5 days. Washed bacteria were inoculated directly into the sand at a concentration of 10^4 cfu ml^{-1} sand following seedling emergence. Control plants were irrigated with tap water or with autoclave-killed bacteria. The sand surface of each pot was covered with a layer of 2-3 cm sterile vermiculite to prevent possible dispersion of bacteria by air currents. Plants were grown in a controlled greenhouse at 25 ± 3°C.

Determination of bacterial numbers associated with roots

Sand was removed and the roots were homogenized in 5 ml PBS by a disperser (Ystral model x 10/20, Germany). Bacteria were enumerated by ELISA and by plate count methods. Plants found to have no detectable colonization when analyzed by ELISA and plate count methods were reanalyzed using the time-limited enrichment technique. The original population size in roots was calculated from the regression lines obtained from liquid cultures.

Statistical analysis

Data were analyzed using a completely randomized design (CRD) with three replicates. A replicate consisted of three test tubes or three Petri dishes or two plants. All experiments were repeated twice. Results from both experiments in each case (total of 6 replicates) were grouped and analyzed together by linear regression ($P \leq 0.01$).

RESULTS AND DISCUSSION

Calibrated regression lines for enumeration of *A. brasilense* are shown in Fig. 1 for strain Cd (A), Somali 67 (B), Sp. 245 (C) and Sp. 246 (D). Lower limits of detection were: $> 10^1$ cfu ml^{-1} for Cd; $>5 \times 10^2$ cfu ml^{-1} for Somali 67 and Sp. 245 and $>5 \times 10^1$ cfu ml^{-1} for Sp. 246.

Because the enrichment procedure is based on bacterial growth, growth variables such as composition of the nutrient solution, bacterial growth phase at inoculation and aeration may affect *Azospirillum* spp multiplication. Figure 2 demonstrates the effect of different media on the growth and detection of *A. brasilense* Cd showing measurable differences between the regression lines. However, the trend of *A. brasilense* Cd growth was similar on all the media tested.

Evaluation of the time-limited enrichment procedure to detect low numbers of *Azospirillum* spp in wheat roots was carried out by comparing it with two other reliable counting methods, MPN and ELISA. Table 1 shows that the enrichment procedure can reveal *A. brasilense* Cd numbers lower than the ELISA detection limit and that the values obtained

Table 1. Enumeration of *A. brasilense* strains in wheat roots using different enumeration methods

Bacterial strain / Root sample No.	Cd			Sp. 245		Sp. 246		Somali 67	
	ELISA*	MPN†	TLLE	MPN	TLLE	MPN	TLLE	MPN	TLLE
				cfu g (fresh wt)[1]					
1	$5x10^4$‖	$8x10^4$	$6x10^4$	$5x10^5$	$4x10^5$	$3x10^5$	$1x10^5$	$1x10^5$	$9x10^4$
2	0§	$2x10^3$	$4x10^3$	$1x10^5$	$9x10^4$	$9x10^4$	1x105	$4x10^4$	$6x10^4$
3	0	$8x10^2$	$3x10^2$	$8x10^4$	$1x10^5$	$8x10^4$	2x105	$9x10^3$	$2x10^4$
4	0	0	$4x10^1$	$6x10^4$	$8x10^4$	$4x10^4$	$8x10^4$	$8x10^3$	$1x10^4$
5&	0	0	0	0	0	0	0	0	0

*According to Levanony *et al.* (1987).

†According to Bashan and Levanony (1985); MPN values according to Postgate (1969).

‖Mean of duplicates.

§Below the level of ELISA detection.

&Non-inoculated plants.

TLLE, time-limited liquid enrichment method.

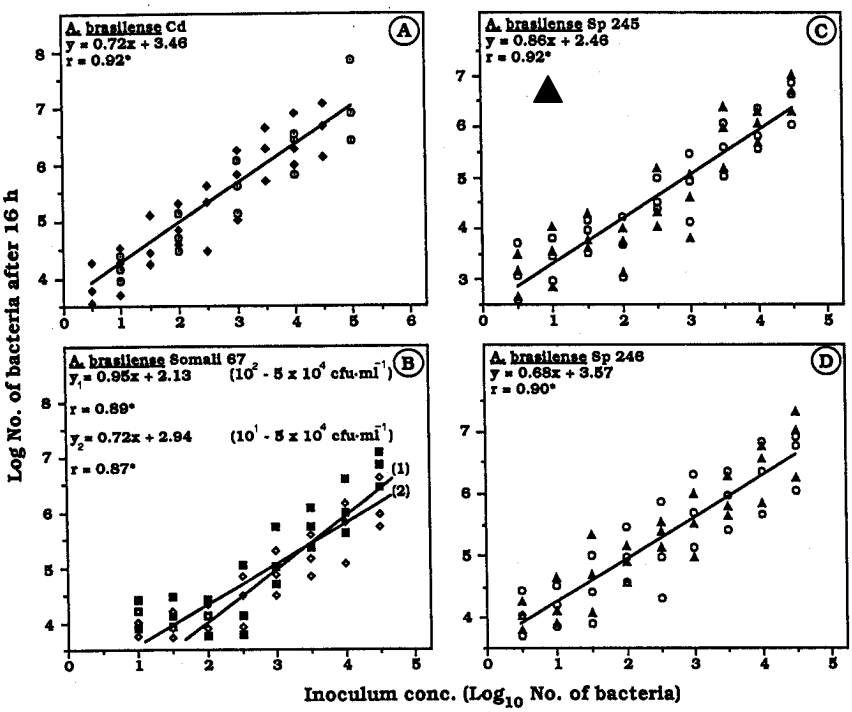

Fig. 1. Enumeration of *A. brasilense* by the time-limited liquid enrichment procedure. (A) Strain Cd; (B) strain Somali 67; (C) strain Sp. 245; (D) strain Sp. 246. Enumeration after bacterial enrichment was detected by: (A) ELISA ( ) and on BL mediium ( ); (B) on OAB medium (, represent two separate experiments), and (C, D) on NFb medium (, represent two separate experiments). *Indicates significance of the regression line at $P \leq 0.01$.

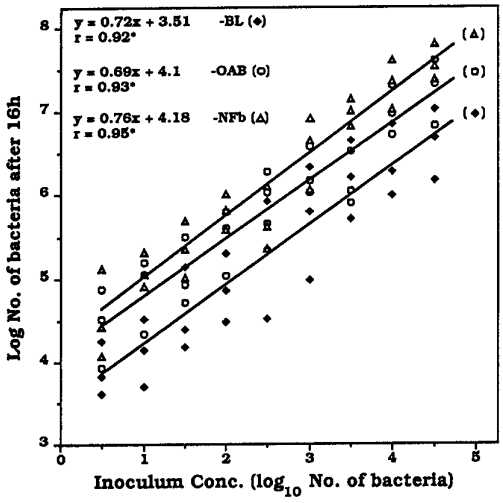

Fig. 2. Effect of three different enrichment media on the enumeration of *A. brasilense* Cd by the time-limited liquid enrichment procedure. Each medium was analyzed separately. *Indicates significance of the regression line at $P \leq 0.01$.

Approximated those obtained with the commonly used MPN technique. However, when high numbers of *Azospirillum* spp are present in the roots there is no need to perform this procedure since ELISA will give an accurate enumeration within 1 day. Similar results were obtained for the other strains using the MPN method (Table 1).

The advantages of the enrichment procedure are: (i) enumeration is much faster, especially if ELISA detection is adopted as the count method of the enriched cultures (~ 30 h compared to 5-8 days for MPN). Additionally, the ELISA enumeration technique is more reliable because it employs specific antibodies for a particular strain; (ii) if a plate count method is used, the proposed procedure saves the use of acetylene reduction assay employed by MPN but will not save time. The disadvantages of the enrichment procedure are: (i) to gain full benefits from this procedure, specific antibodies should be produced for every strain. Presently, specific antibodies are available for only a limited number of strains (Levanony *et al.*, 1987; Schank *et al.*, 1979); (ii) the enrichment procedure is very sensitive to variations in culture media composition and to sampling time from the semi-solid medium. Therefore, it is advisable that the regression lines demonstrated in this study should be considered as guidelines only, and the enrichment procedure only used after a calibration line has been produced for each given strain and medium used.

In conclusion, our study presents a simple enrichment procedure to aid in the quantitative detection of very low numbers of *A. brasilense* in plant roots, which can be used as a complementary procedure to ELISA and MPN techniques.

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