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WEST PALAEARCTIC REGIONAL SECTION

ENUMERATION AND IDENTIFICATION OF RHIZOSPHERE BACTERIA BY ADVANCED IMMUNO TECHNIQUES

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ABSTRACT

This study describes the technical details of five immuno techniques used in our laboratories for monitoring the *Azospirillum* colonization of roots.

INTRODUCTION

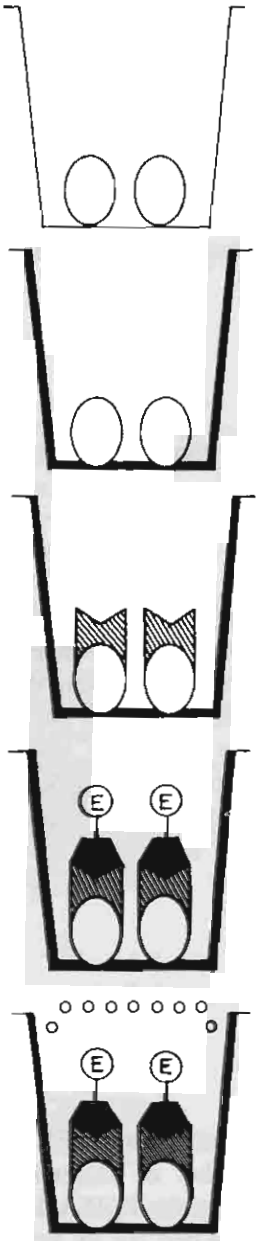
There has always been a need in plant rhizosphere studies to develop rapid, simple assay methods counting rhizosphere bacteria in a reproducible manner. Selective media are not very efficient. Whenever a particular rhizosphere strain was applied to the rhizosphere, it was not possible to monitor it reliably because of a background of other unidentified rhizosphere bacteria capable of growing on the medium (1). Thus, more reliable methods are essential for identification and enumeration of the applied strain, and for the evaluation of its colonization ability in the rhizosphere.

MATERIALS AND METHODS

Those were described in the papers cited in the reference list (1, 2, 3, 4, 5, 6).

RESULTS

Indirect and competition enzyme-linked immunosorbent assays (ELISA) were employed for bacterial quantification when the number of bacteria per sample exceeded 10^5 CFU (Figs. 1 and 2) (5). Amplification of the ELISA detection level at its lower marginal level ($<10^6$ CFU/ml) was carried out by avidin-biotin ELISA (Fig. 3) (4). When the number of bacteria in the samples was smaller than 10^4 CFU, the time-limited liquid enrichment technique combined with ELISA was used (Table 1) (2). Ultrastructural identification of *Azospirillum brasilense* Cd, on and within wheat roots, used the immuno-gold labeling technique (Fig. 4) (6).



1. Bacteria adsorbed to well surface in 0.05 M sodium carbonate buffer, pH 9.6 + 0.02 % Azide, 4 °C. 16-18 hr, 10^5-$5x10^8$ CFU/ml.

Wash (x3) PBS + 0.05 % Tween 20 (PBST) (3 min intervals)

2. Blocking with 1 % egg albumin in PBST, 37 °C, 1 hr.

Washed as above

3. Binding of polyclonal specific antibody, 1:100 in PBST + 0.02 % Azide (PBSTA), 37 °C, 90 min.

Washed as above.

4. Goat-antirabbit antibody linked to alkaline phosphatase. 1:5000 in PBSTA + 2 % polyvinylpyrrolidone + 0.2 % bovine serum albumin. 4 °C. 16-18 hr.

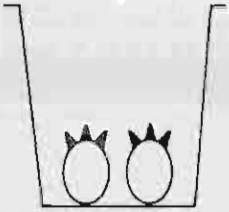
Washed as above.

5. Substrate added (fresh disodium paranitrophenylphosphate, 0.1 mg/ml) in diethanolamine buffer, pH 9.8 + 0.05 % Azide.

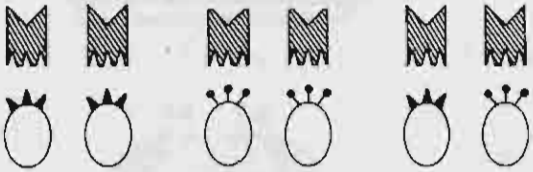
6. Incubation at 37 °C, 2-24 hr.

7. Color intensity OD_{405} = bacterial number.

Fig. 1. Schematic representation of the indirect-ELISA procedure.



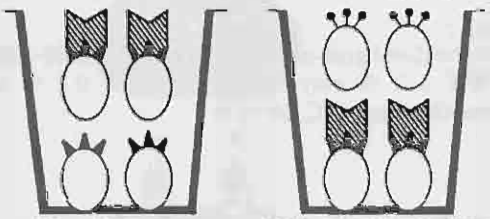
Wash (x3) PBS + 0.05 % Tween 20 (PBST) (3 min intervals).



Competitor bacteria (Bacteria with common antigens like the adsorbed bacteria).

Non-competitor bacteria (Bacteria having no common antigens with the adsorbed bacteria).

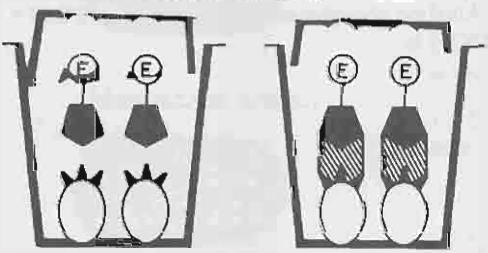
Mixture of competitor + non-competitor bacteria.



With competitors

With non-competitors

Washed as above



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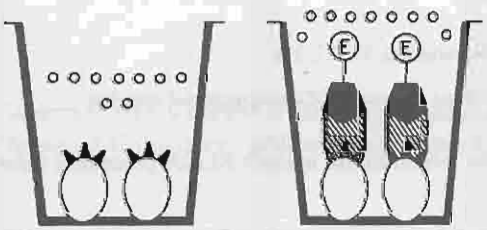


1. Bacteria adsorbed to well surface in 0.05 M sodium carbonate buffer, pH 9.6 + 0.02 % Azide, 4 °C, 16-18 hr, ($5x10^7$ CFU/ml).

2. Blocking as in indirect ELISA.

3. Polyclonal specifie antibody (1:1000 in PBSTA) mixed with bacterial competitors, 90 min, 37 °C in glass tubes.

4. Mixture of antibody + competitor/non-competitor attached, 37 °C, 90 min.

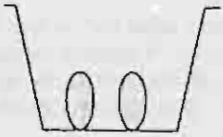
5. Goat-antirabbit antibody coupled to alkaline phosphatase 1:5000 in PBSTA + 2 % polyvinylpyrrolidone + bovine serum albumine, 4 °C, 16-18 hr.

6. Substrate added (fresh disodium paranitrophenyl phosphate, 0.1 mg/ml) in diethanolamine buffer, pH 9.8 + 0.05 % Azide.

7. Incnbation at 37 °C, 2-24 hr.

8. Color intensity OD_{405} is inversely related to the number of bacteria in the sample.

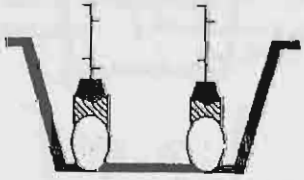
Fig. 2. Schematic representation of the competition-ELISA procedure.



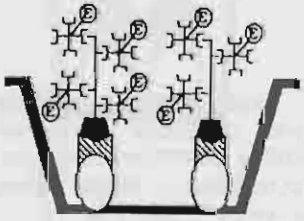
1. Bacteria adsorbed to well surface in 0.05 M sodium carbonate buffer, pH 9.6 + 0.02 % Azide, 4 °C, 16-18 hr, 10^4-10^8 CFU/ml.

2. Blocking with 1 % egg albumin in PBST, 37 °C, 1 hr.

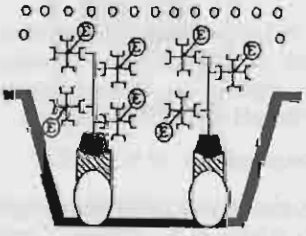
3. Polyclonal specific antibody attached, 1:1000 in PBST + 0.02 % Azide (PBSTA), 37 °C, 90 min.



4. Biotinylated goat-antirabbit antibody, 1:1000-1:2000 in PBSTA + 2 % polyvinylpyrrolidone + 0.2 % bovine serum albumine, 4 °C, 16-18 hr.



5. Alkaline phosphatase labeled avidin in PBSTA 1:1000, 37 °C, 2 hr.

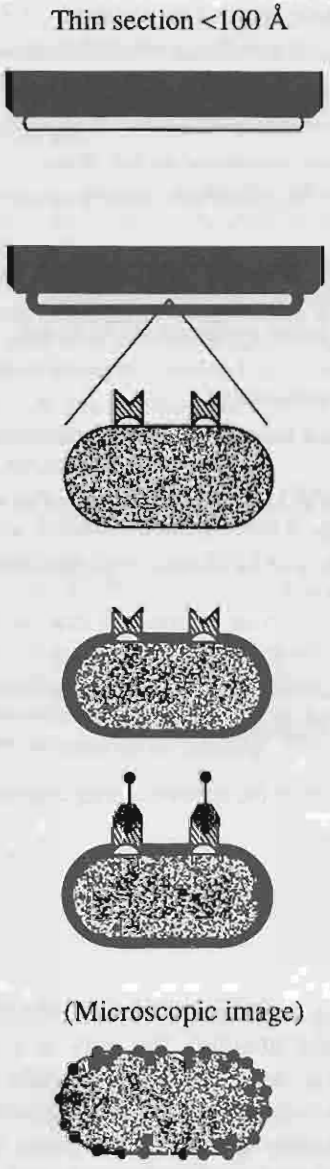


6. Substrate added in 10 % diethanolamine buffer pH 9.8 + 0.05 % Azide.

7. Incubation 37 °C, 2 hr.

8. Color intensity OD_{405}=bacterial number.

Fig. 3. Schematic representation of the avidin-biotin indirect-ELISA procedure (after Levanony and Bashan, 1990).



1. Oxidizing with 10 % H_2O_2 for 10 min.

2. Rinse in double distilled water.

3. Blocking with 1 % egg albumin in PBS (pH 7.2) + 0.05 % Tween 20 + 20 mM Azide for 15 min.

4. Blot excess solution

5. Polyclonal specific antibody attached. 1:1000 in PBS for 90 min.

6. Rinse in PBS.

7. Blocking as above for 4 min.

8. Blot excess solution.

9. Goat anti-rabbit antibody conjugated to colloidal gold. Diluted 1:10 in Tris buffered saline (pH 7.4) for 30-60 min.

10. Rinse in PBS and then in distilled water.

11. Stain with uranyl-acetate and lead citrate.

12. Observation under transmission electron microscope.

Fig. 4. Schematic representation of the immuno-gold labeling procedure. All solutions were filtered through 0.45 μm filters; grids with section down.

Table 1. Time-limited liquid enrichment technique for *Azospirillum*.

Production of regression analysis growth curves for *Azospirillum* strains
1. Grow *Azospirillum* strains in any rich medium until exponential growth phase.
2. Wash in PBS. Decimal dilution of the bacteria in PBS (phosphate-buffered saline) to 10^1 CFU/ml (verification by a plate count method).
3. Inoculate 0.1 ml samples (in triplicates, in gradually increased concentrations of 10^1 10^5 CFU/ml) into any N-free, semi-solid (0.05% agar) medium in test tubes.
4. Stir once and grow the bacteria at 30 °C without further movement for 16 hr only.
4a. A preliminary step. Steps 1-4 followed by incubation of 4-8 days is required to detect the depth (in mm) of the pellicle formed by each *Azospirillum* strain.
5. Samples are taken from the predicted site of pellicle formation after 16 hr (no visible sign of pellicle at this time).
6. Bacteria in the samples are identified and counted by any ELISA technique or by a plate count method (in the absence of specific antibodies).
7. Draw a regression line for each strain (inoculation level vs. number of bacteria developed after 16 hr in the pellicle site).
Bacteria counts from roots
1. Homogenize root samples having less than 10^5 CFU/sample.
2. Perform steps 3, 4, 5 and 6.
3. The original population size in roots is calculated from the regression lines obtained from the liquid cultures.

DISCUSSION

These techniques are being used mainly in clinical research (ELISA techniques) and in cell physiology (immuno-gold labeling), but only to a marginal extent in soil microbiology. The procedures described were modified to suit studies in the multi-species organisms environment of the rhizosphere. These techniques are simple, versatile, accurate, highly specific at the strain level, reproducible, inexpensive, consume relatively short working periods and are suitable for large-scale testing of plant-bacteria samples.

ACKNOWLEDGMENTS

This study is dedicated to the memory of the late Mr. Avner Bashan. All artwork was carried out by Roberto Lomeli-Leos from the Center of Biological Research, La Paz, Mexico. We thank Roy Bowers, the University of Baja California

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