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Improved Medium for Isolation of *Azospirillum* spp.

ENRIQUE A. RODRÍGUEZ CÁCERES

Departamento de Microbiología, Instituto Nacional de Tecnología, Agropecuaria, 1712 Castelar, Buenos Aires, Argentina

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Colonies of *Azospirillum* spp. could be readily distinguished from colonies of other diazotrophs by scarlet coloration in culture media in which Congo red was included.

Azospirillum spp. are among the most important bacteria involved in N_2 fixation in grasses. It is normal for *Azospirillum* spp. to be isolated from nitrogen-free culture media inoculated with soil or roots. However, Döbereiner et al. (2) have reported that isolation of pure cultures from samples collected in temperate regions is difficult. Vlassak and Reynders (6) have found that in many cases, nitrogen-fixing bacteria are closely associated with other microorganisms and that obtaining pure isolates is not easy.

The use of Congo red for distinguishing between rhizobia and other bacteria is well known (3). This paper describes a simple technique which permits the recognition of *Azospirillum* colonies on plates and facilitates the isolation of pure cultures.

Two media were employed: nitrogen-free semisolid malate (NFb) enrichment medium (1), and Rojo Congo (Congo red; RC), an isolation medium. On the basis of the chemical composition of medium 79 (3), several carbon sources for *Azospirillum* spp. and concentrations of yeast extract (0 to 1 g · liter^{-1}) were used in RC medium, which also contained the following (grams per liter of distilled water): K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; yeast extract, 0.5; $FeCl_3 \cdot 6H_2O$, 0.015; DL-malic acid, 5; KOH, 4.8; and agar, 20. The pH was adjusted to 7.0 with 0.1 N KOH, and the medium was autoclaved at 121°C for 20 min.

I added 15 ml of a 1:400 aqueous solution of Congo red (autoclaved separately) aseptically to each liter of the melted medium just before tubing or use.

Samples of soil from corn (*Zea mays* L.), sorghum (*Sorghum* sp.), and rice (*Oryza sativa* L.) crops, collected in areas of Argentina, or root pieces from the same species (washed with sterile water or treated with 1% chloramine T [4]) were placed into tubes, each of which contained 5 ml of NFb medium. These enrichment cultures were incubated at 37°C for 72 h. A white, dense, undulating, diffuse pellicle was then observed 1 to 4 mm below the surface. Examination of wet mounts by phase-contrast microscopy revealed, among other microorganisms, rods with fat droplets and active movements characteristic of *Azospirillum* spp. Posi-

FIG. 1. Isolation plates of RC medium. Incubation was at 37°C for 72 h. *Azospirillum* colonies were easily recognized.

FIG. 2. First streaks showing small *Azospirillum* colonies among the contaminants.

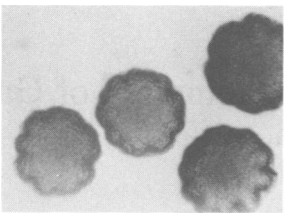

FIG. 3. *Azospirillum* colonies after 96 h of incubation. Magnification, ×10.

tive cultures were serially diluted 10-fold in sterile tap water to 10^{-4} to 10^{-5}. Loopfuls of the dilutions were streaked on plates of RC medium, which were incubated at 37°C for 72 h.

Light-pink and colorless colonies were observed after 48 h. After 72 h, the light-pink colonies became scarlet (Fig. 1). Small scarlet colonies were observed in the first streaks, indicating the presence of *Azospirillum* spp. among the contaminants (Fig. 2). Phase-contrast microscopic examination of wet mounts of the scarlet colonies revealed rods resembling *Azospirillum* cells.

The colonies were diluted in sterile tap water, and the dilutions were streaked on plates of RC medium to check the purity of the isolates. Uniformity of colony color was observed.

Diagnostic biochemical and physiological tests were conducted by the method of Tarrand et al. (5) and confirmed the identity of the strains.

To confirm the usefulness of this technique, I streaked a loopful of an *Azospirillum brasilense* culture from the American Type Culture Collection (ATCC 29145) on RC medium. All colonies became scarlet after incubation.

Two species from roots and soil samples were identified on the basis of the capacity to use glucose as the sole carbon source for growth in NFb medium containing biotin (5): *Azospirillum lipoferum* and *A. brasilense*. Scarlet colonies of both species grew in plates of this medium. Colonies that developed in petri dishes of RC medium incubated at 37°C for 96 h had the following characteristics: scarlet color, abundant growth, dry consistency, diameter of 1.5 to 2 mm, round or irregular form, undulate edge, and rugose surface with ridges radiating from the center (Fig. 3). The colonies of other root-associated bacteria (species of *Bacillus, Enterobacter, Klebsiella* and *Pseudomonas*) are circular, convex, translucent, and smooth, have an entire margin, and do not absorb Congo red.

Many pure cultures were readily obtained by this method.

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