

Selective Medium for Recovering Specific Populations of Rhizobia Introduced into Tropical Soils†

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Experiments were designed to evaluate the usefulness of antifungal agents and streptomycin for recovering low densities of rhizobia inoculated into tropical soils. The results showed that yeast-mannitol agar (pH 6.0) containing 500 µg of streptomycin, 400 µg of cycloheximide and 50 µg of benomyl or chlorothalonil per ml was the best selective medium.

Ever since Obaton (7) proposed the use of spontaneous mutants resistant to antibiotics for ecological studies of rhizobia, selective media containing streptomycin and the antifungal agent cycloheximide have been employed for enumerating rhizobial populations added to soil (2-4, 8). However, this approach is inadequate for studies involving low densities of rhizobia, especially when the rhizobia are slow-growing ones (1, 2, 6). This inadequacy arises largely from the interference of fast-growing and spreading fungi

1-liter screw-cap jars at a moisture content of about 60% available water-holding capacity. The soils had pHs of 4.9, 6.2, 4.2, 4.5, 5.7, and 6.0, respectively.

Cycloheximide, griseofulvin, nystatin, pimaricin, and streptomycin were purchased from Sigma Chemical Co., St. Louis, Mo. Benomyl, captan, mancozeb, and thiram were supplied by E. I. du Pont de Nemours and Co., Wilmington, Del. Chlorothalonil was supplied by M. Aragaki, Department of Plant Pathology, University of Hawaii, Honolulu.

TABLE 1. Recovery of rhizobial cells of indicated strains from culture suspensions on YMA containing streptomycin and cycloheximide with or without a second fungicide

| Second fungicide | Cells (10 ⁶)/ml in the following strains | | | | | | | |
|-------------------------|--|-----------|-----------|----------------|------------|------------|-------|----------------|
| | 1SR | Tal 209SR | Tal 582SR | Tal 659SR | Tal 1145SR | Tal 3176SR | J-1SR | SC7SR |
| None | 2,800 | 4,300 | 2,500 | 2,800 | 840 | 690 | 1,900 | 900 |
| Benomyl | 2,600 | 2,500 | 2,500 | 1,400 | 830 | 220 | 920 | 490 |
| Chlorothalonil | 1,600 | 2,200 | 2,400 | 2,000 | 770 | 340 | 970 | 590 |
| Captan | 250 | 120 | 51 | — ^a | 1.9 | 23 | 1.8 | 50 |
| Griseofulvin | 600 | 4,400 | 3,000 | 330 | 860 | 680 | 620 | — ^a |
| Mancozeb | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nystatin | 820 | 4,100 | 2,200 | 350 | 810 | 730 | 350 | — ^a |
| Thiram | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| LSD (0.05) ^b | 320 | 470 | 350 | 310 | 77 | 75 | 260 | 150 |

^a —, Not determined.

^b LSD, Least significant difference.

when soil suspensions below a 10⁻⁵ dilution are used. In many soils of Hawaii, fungi that can grow and spread rapidly on yeast-mannitol agar (YMA) amended with streptomycin and cycloheximide are abundant. Some of these fungi are also known to suppress the development of rhizobial colonies through the production of toxic metabolites (5). The purpose of this investigation was to develop a selective medium based on YMA that will be useful for studying the ecology of low densities of rhizobia in different tropical soils.

The soils used in this study were surface soil samples (0 to 15 cm) from the Hanalei series (Typic Torrifluent), the Keahua series (Toroxic Haplustoll), the Lahaina series (Tropoctic Haplustox), the Paaloa series (Humoxic Tropohumult), the Wahiawa series (Tropoctic Eustrustox), and the Waialua series (Vertic Haplustoll). The soil samples were crushed to pass through a 2.0-mm sieve and stored in

YMA (9) was used for maintaining and culturing rhizobia. YMA at pH 6.0 containing 500 µg of streptomycin and 400 µg of cycloheximide per ml with or without 50 µg of a second fungicide was used to test the sensitivity of pure cultures of streptomycin-resistant rhizobia and soil fungi and to recover rhizobia from inoculated soil.

Rhizobium sp. strain 1 was isolated from nodules of *Sesbania grandiflora* grown on the Waialua soil. *Rhizobium* sp. strains J-1 and SC7 were obtained from A. Eaglesham and H. Lowendorf, respectively, of Cornell University, Ithaca, N.Y. *R. phaseoli* strains Tal 659 and *Rhizobium* sp. strains Tal 209, Tal 582 and Tal 659 were obtained from the culture collection of the NifTAL Project, University of Hawaii, Maui. Spontaneous mutants of the cultures that are resistant to 500 µg of streptomycin per ml were obtained by growing the cultures for 4 to 10 days on YMA slants at 30°C, suspending the cells in 10 ml of saline (8), inoculating 1.0 ml of the 10⁻¹ to 10⁻⁷ dilution suspension into sterile petri plates, and pour plating with YMA contain-

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TABLE 2. Suppression of soil fungi on YMA containing streptomycin and cycloheximide with or without a second fungicide

| Second fungicide | No. of CFU/plate at a 10 ⁻³ dilution of indicated soil ^a | | | | | |
|------------------|--|---------|---------|-----------|---------|---------|
| | Hanalei | Lahaina | Keahua | Paaloa | Wahiawa | Waialua |
| None | 57 ± 4 | 38 ± 6 | 52 ± 9 | Confluent | 52 ± 21 | 58 ± 14 |
| Benomyl | 13 ± 4 | 2 ± 1 | 0 ± 0 | 0 ± 0 | 4 ± 2 | 0 ± 0 |
| Chlorothalonil | 0 ± 0 | 0 ± 0 | 1 ± 0 | 0 ± 0 | 11 ± 3 | 0 ± 0 |
| Griseofulvin | 54 ± 11 | 27 ± 4 | 49 ± 15 | Confluent | 74 ± 17 | 46 ± 2 |
| Nystatin | 41 ± 14 | 15 ± 4 | 46 ± 5 | Confluent | 43 ± 1 | 25 ± 2 |
| Pimaricin | 26 ± 8 | 36 ± 4 | 33 ± 3 | Confluent | 39 ± 6 | 8 ± 1 |

^a Values represent means of duplicate observations plus or minus 1 standard deviation.

ing 500 µg of streptomycin per ml. Plates were incubated at 30°C in the dark until colonies were well developed. Individual colonies were picked up and maintained on YMA slants as streptomycin-resistant mutants.

Serial dilutions of 4- to 10-day-old slant cultures of rhizobia or the moist test soils (60% available water-holding capacity) were prepared, and the number of CFU developing on YMA containing 500 µg of streptomycin and 400 µg of cycloheximide per ml of medium with or without 50 or 250 µg of a second fungicide was determined. Fungal suppression was considered effective when CFU failed to develop on plates receiving soil suspensions from a 10⁻³ dilution or when the number of fungal CFU 6 days after plating was less than 20 and occupied 15% or less of the surface of the plating medium after 12 days. The area occupied by fungal CFU, in most instances, was determined by making a photocopy of the bottom of the plate, cutting out the area of the paper covered by the colonies, weighing it, and determining the area it occupied from a standard curve of weight versus area prepared with a piece of paper identical to the one used for photocopying the plate. In situations in which colonies were few or too many and small in size, areas were calculated after the diameters of the colonies were measured. Each determination was replicated two times.

The usefulness of the selective medium of choice for studying the ecology of *Rhizobium* strains was tested in an experiment involving the survival of *Rhizobium sp.* strain 1SR in the Wahiawa soil (pH 4.2). For this purpose, a 4-day-old culture of the rhizobium grown in yeast-mannitol broth (9) was harvested by centrifugation at 8,000 X g and washed twice in saline solution; the pellet was brought back to original volume with saline solution. To 25-g portions of the Wahiawa soil contained in three 100-ml screw-cap jars, 2.5-ml portions of a 10-fold dilution of the washed cell suspension of the rhizobium were added. The moisture content of the soil was adjusted to about 60% available water-holding capacity. Jars were incubated at 30°C in the dark. At regular intervals, 1.0-g samples were withdrawn,

serial dilutions of the samples were prepared, and the numbers of rhizobia surviving were determined on YMA containing 400 µg of cycloheximide, 500 µg of streptomycin, and 50 µg of benomyl or chlorothalonil per ml of medium.

When cell suspensions of rhizobial strains that were resistant to 500 µg of streptomycin and 400 µg of cycloheximide per ml were plated out on YMA (pH 6.0) containing streptomycin and cycloheximide with or without a second fungicide, the effect of the chemicals was variable, depending on the chemical used and the strain of *Rhizobium* tested (Table 1). Captan, mancozeb, and thiram were 92 to 100% inhibitory to the rhizobia. The bacteria were either inherently resistant or only partially inhibited by benomyl, chlorothalonil, griseofulvin, and nystatin. However, only benomyl and chlorothalonil were able to effectively suppress the development and spread of fungi when suspensions from six different soils were plated on YMA containing streptomycin and cycloheximide with one of the latter four fungicides (Tables 2 and 3). When soil suspensions from the Hanalei or the Wahiawa soil were plated on YMA containing streptomycin with 25 or 50 µg each of benomyl and chlorothalonil, the results obtained were variable. Fungi from the Wahiawa soil covered 36 or 58% of the surface of YMA after 12 days. On two of the four plates containing the above-mentioned chemicals, development of fungi from the Hanalei soil was completely suppressed, whereas 83 to 95% of the surfaces of the remaining two plates were covered with resistant fungi after 12 days. The effect of the fungicide combinations was similar, regardless of the concentrations tested. On the basis of these observations, YMA (pH 6.0) containing 500 µg of streptomycin, 400 µg of cycloheximide, and 50 µg of benomyl or chlorothalonil was chosen as the best selective medium for recovering rhizobia introduced into tropical soils.

Figure 1 illustrates the results obtained when the proposed selective medium was used to recover *Rhizobium sp.* strain Tal 582SR inoculated into the Wahiawa soil. The results clearly demonstrate that the medium could be very useful for

TABLE 3. Suppression of soil fungi on YMA containing streptomycin and cycloheximide with or without a second fungicide

| Second fungicide | Occupation of YMA surface by fungi at a 10 ⁻³ dilution of indicated soil (%) ^a | | | | | |
|------------------|--|---------|---------|---------|---------|---------|
| | Hanalei | Lahaina | Keahua | Paaloa | Wahiawa | Waialua |
| None | 89 ± 15 | 48 ± 12 | 77 ± 33 | 100 ± 0 | 90 ± 15 | 28 ± 8 |
| Benomyl | 82 ± 26 | 2 ± 1 | 0 ± 0 | 0 ± 0 | 4 ± 2 | 0 ± 0 |
| Chlorothalonil | 0 ± 0 | 0 ± 0 | 2 ± 1 | 0 ± 0 | 4 ± 1 | 0 ± 0 |
| Griseofulvin | 84 ± 6 | 37 ± 11 | 49 ± 15 | 100 ± 0 | 67 ± 2 | 14 ± 3 |
| Nystatin | 55 ± 3 | 42 ± 6 | 90 ± 9 | 100 ± 0 | 80 ± 20 | 60 ± 27 |
| Pimaricin | 85 ± 14 | 83 ± 20 | 82 ± 6 | 100 ± 0 | 81 ± 3 | 12 ± 3 |

^a Values represent means of duplicate observations plus or minus 1 standard deviation.

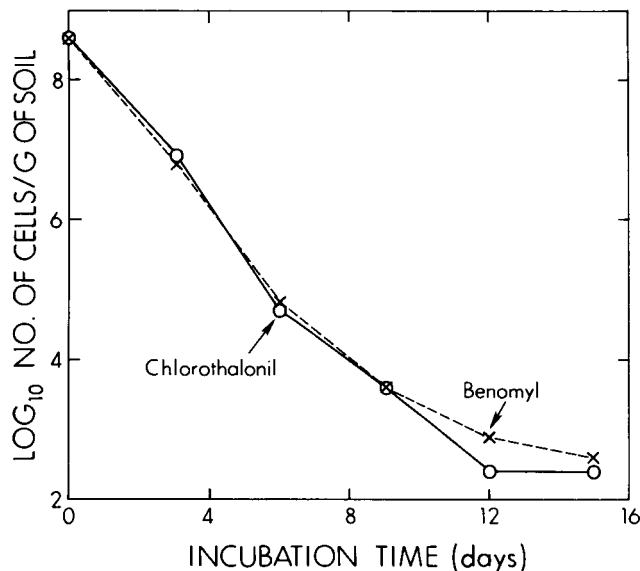


FIG. 1. Recovery of *Rhizobium* sp. strain 1SR from the Wahiawa soil on YMA containing streptomycin, cycloheximide, and benomyl or chlorothalonil. Recovery values observed at each sampling period are not statistically different from each other at the 5% level.

studying the ecology of low densities of specific populations of rhizobia in tropical soils. The ability of the medium to effectively suppress interfering fungi from soils widely differing in their chemical and physical properties for up to 12 days suggests that it is suitable for studying the ecology of both slow- and fast-growing rhizobia in various soils. Recently, Bushby (1) proposed a selective medium which he considered to be effective for the quantitative estimation of low numbers of slow-growing rhizobia in soils. In this medium, interfering fungi from the one Australian soil tested were suppressed by cycloheximide and pimafucin (pimaricin), a combination of fungicides which failed to effectively suppress the development of fungi from five of the six soils tested in the current investigation (Table 3). It thus appears that the effectiveness of a selective medium must be evaluated with several soils before its widespread usefulness can be presumed.

With some of the soils tested, there is a lack of correspondence between the effect of the fungicides on fungal abundance and their effect on the subsequent spread of fungal CFU. This is illustrated by the effect of griseofulvin on fungi from the Waiialua soil and the effect of benomyl on fungi

from the Hanalei soil. In the former case, the fungicide was effective in suppressing fungal spread without being very effective in restricting fungal abundance. In situations like this, there will be many small fungal CFU which will be difficult to distinguish from rhizobial colonies unless plates are viewed in daylight and the target colonies are marked. This requirement makes the counting process awkward and difficult. The effect of benomyl on fungi from the Hanalei soil, on the other hand, was to restrict the abundance of fungi on the plate without effectively suppressing their subsequent spread, thus making rhizobial counts altogether impossible. The best antifungal agent is, therefore, one that effectively restricts both the abundance and the spread of fungal CFU. It has been my experience that the presence of fungi will not invalidate or make rhizobial counts too difficult as long as the number of fungal CFU does not exceed 20 and the area these CFU occupy is not in excess of 15% of the surface of YMA medium. These observations stress the need to determine both the abundance and spread of fungal CFU when evaluating the usefulness of antifungal agents in selective media.

It should be remembered that not all of the rhizobial strains tested were completely resistant to benomyl or chlorothalonil, and in cases in which test populations are not 100% resistant, the cultures should be preexposed to the fungicides to eliminate the sensitive fractions of the populations before ecological studies are initiated.

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