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SHORT COMMUNICATION

Rapid method to enumerate and isolate soil actinomycetes antagonistic towards rhizobia

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Inhibition of rhizobia by actinomycetes in soils has been suggested as a cause of the failure of legume inoculation (Van Schreven, 1964; Damirgi and Johnson, 1966; Patel, 1974; Foo and Varma, 1976). Generally, antagonistic activity against a given *Rhizobium* strain is determined according to a two-step procedure. Actinomycetes are first isolated by selecting colonies from agar plates made from diluted soil samples. Their antagonism to *Rhizobium* is tested on an agar medium suitable for both actinomycetes and *Rhizobium* (Visona and Tardieux, 1964; Hattingh and Louw, 1966). Unfortunately this method is very time consuming.

We describe a selective triple-agar layer technique developed to simultaneously enumerate and isolate soil actinomycetes antagonistic to slow- or fast-growing *Rhizobium* strains. In order to achieve this aim, actinomycetes had to be selectively stimulated and the spread of bacteria and fungi had to be prevented. Usually bacteria are suppressed by adding antibiotics to the medium (Williams and Cross, 1971), but addition of antibacterials would affect the growth of the *Rhizobium* strain being tested. Therefore phenol, which was used here as an antibacterial, was added to the soil suspension but not to the isolation medium (Lawrence, 1956). Fungi were controlled by adding cycloheximide to the medium. Since antagonism was assayed directly on the soil dilution plate, transferring isolates for streaking tests was not necessary.

Bottom layer

Soil (10 g) was ground in a sterile mortar with a little water and transferred to a dilution blank prepared by dissolving 631 mg phenol in 90 ml sterile water. This suspension was then shaken for 10 min on a rotary motion shaker (220 cycles min^{-1}). Dilutions were made and incorporated in 100 ml of glycerol-arginine-salt medium at 40–45°C. This is a modification of the medium for preferential isolation of actinomycetes described by Porter *et al.* (1960): pH was adjusted to 6.5 instead of 7.0 and cycloheximide, sterilized by filtration (Millipore 0.22 μm), was added at a final concentration of 200 mg l^{-1}. The soil suspension agar medium was distributed in 10 replicate plates, 10 ml of medium per plate, and allowed to cool until the agar was firm.

Intermediate layer

An intermediate layer consisting of 5 ml 2% sterile water agar was poured over the bottom layer of each plate. When slow-growing *Rhizobium* had to be tested, the plates with the two layers (bottom and intermediate) were incubated at 26°C for 3 days. But in the case of fast-growing *Rhizobium*, incubation was increased to 6 days to permit sufficient development of actinomycetes before *Rhizobium* growth began.

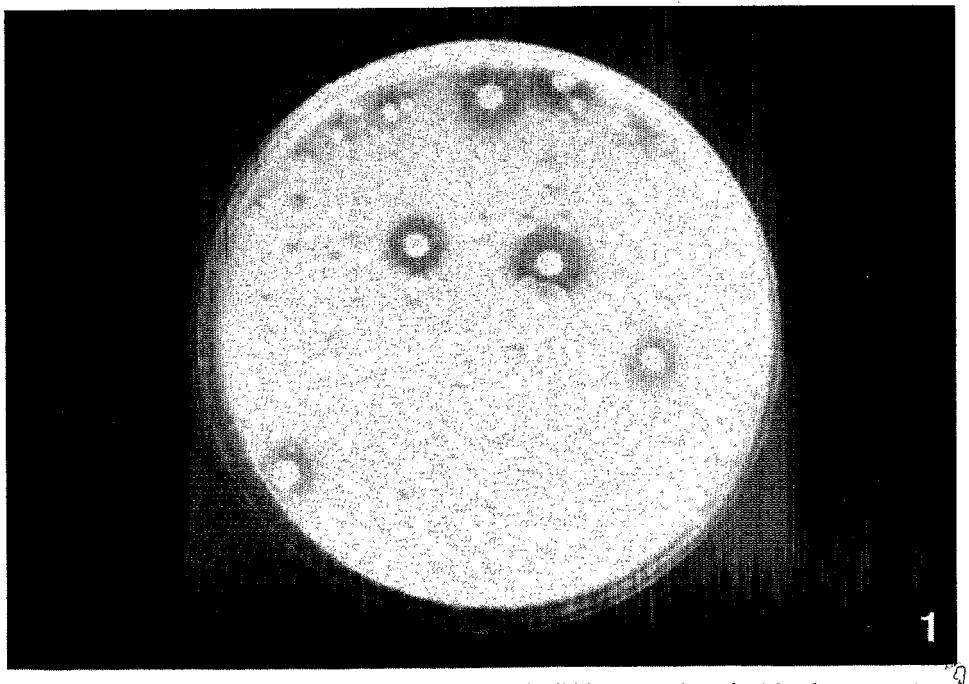

Fig. 1. An assay plate (10 cm dia) exhibiting zones of inhibition; tested strain *Rhizobium japonicum* G_2Sp.

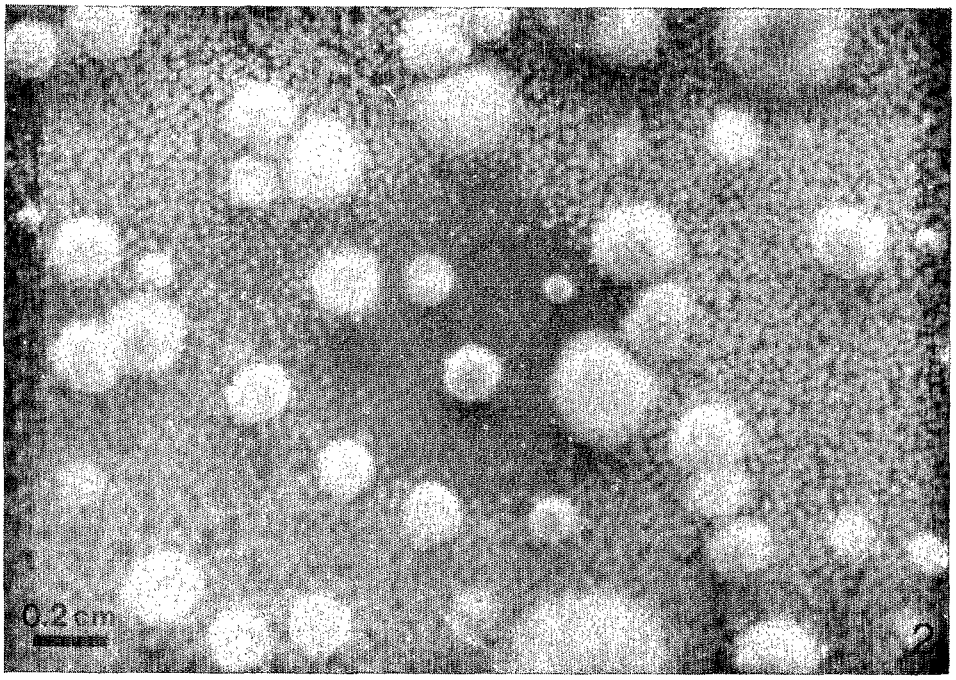

Fig. 2. Enlargement of an inhibition zone surrounding *Rhizobium meliloti* U-45. Larger colonies are actinomycetes; smaller ones are *Rhizobium*. On the picture, only one actinomycete colony appears to be antagonistic towards the tested *Rhizobium* strain.

Top layer

After incubation at 26°C (3 or 6 days according to the type of *Rhizobium* strain), 10 ml mannitol-yeast extract-salt medium for cultivation of *Rhizobium* (Wacek and Brill, 1976) was poured on to the intermediate layer of each plate. When this upper layer was firm, a suspension of the *Rhizobium* strain being tested was sprayed using the Hauduroy apparatus (Hauduroy, 1949) on to the surface of the medium. The plates were incubated at 30°C, which is a favorable temperature for rhizobia growth.

Seven days (slow-growing) or three days (fast-growing) after inoculation inhibition zones surrounding antagonistic actinomycete colonies were visible (Figs 1 and 2). Counts were made and colonies of interest were subcultured on the glycerol-arginine salt medium. Very few bacterial (none of the spreading types) and no fungal contaminants developed in the dilution plates. Nevertheless, when studying soils inhabitated by large fungal populations, it might be necessary to supplement the soil dilution agar medium with a combination of antifungal substances, such as nystatin and pimaricin.

Examples of the utilization of this method are given in Tables 1 and 2. In these applications, made with tropical psamment soils, the final dilutions of soil in the bottom

Table 1. Numbers of actinomycetes occurring in a sample of a tropical psamment soil from Senegal (Dior, Bambey) exhibiting antagonism towards three strains of *Rhizobium*

Rhizobium strain	Total number of actinomycetes g^{-1} dry soil	Number of antagonistic actinomycetes g^{-1} dry soil
Rhizobium japonicum G_2Sp*	8.0×10^4	9.6×10^2
Rhizobium sp. CB-756	8.4×10^4	8.6×10^2
Rhizobium meliloti U-45	7.0×10^4	8.3×10^2

* Streptomycin-resistant mutant of strain 331.b.135. USDA, Beltsville.

Table 2. Numbers of actinomycetes in three samples of tropical psamment soils from Senegal exhibiting antagonism towards *Rhizobium japonicum* G_2Sp

Soil location	Total number of actinomycetes g^{-1} dry soil	Number of antagonistic actinomycetes g^{-1} dry soil
Bel-Air	1.5×10^5	21×10^3
Tiénaba	1.6×10^5	9.2×10^3
Nioro de Rip	3.4×10^5	5.2×10^3

layer were 10^{-4} to 10^{-5}. Hopefully, this simple and rapid technique will facilitate studies on the antagonism of actinomycetes towards rhizobia and could be extended to test a large number of microorganisms including plant-pathogens, since any bacteria or fungi may be sprayed on to the top medium.

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