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Note

A technique for isolating bacteria-free *Pythium* spp. from pond water

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A special technique for isolating aquatic *Pythium* spp. that are free from bacteria is presented and discussed by citing the disadvantages of the previous studies.

Key Words——bacteria-free; new technique; *Pythium* spp.

Isolation of *Pythium* spp. from aquatic habitats by usual laboratory methods (Pittis and Colhoun, 1984) was generally unsatisfactory. A thin film of bacterial contamination usually accompanied the fungal mycelia and acted as a barrier to purification. The use of different selective media for purification (Plaats-Niterink, 1975) also gave a limited result. In the course of investigations into isolation of aquatic pythia from pond waters (Abdelzaher et al., 1994; Abdelzaher et al., 1994a, b; Awad et al., 1993a, b), a special technique which faccilitated the isolation of bacteria-free *Pythium* was developed. This method can also be used for purification of many species of *Pythium* isolated from any source. The method has the advantage of yielding isolates of *Pythium* that are free from bacterial contamination. It also provides a simple and useful method of separating bacteria from *Pythium* spp. and from the other members of the Mastigomycotina.

Although different methods are used for obtaining *Pythium* spp. free from bacterial contamination (Sleeth, 1945; Plaats-Niterink, 1975; Paul and Baghdadi, 1985; Paul, 1968a, b), the present method (Fig. 1) was found to be the best among them.

In two years of isolating *Pythium* spp. from pond water, about 1,000 isolates have been obtained in pure cultures by the above technique without any bacterial contamination. This technique proved to be satisfactory for isolating pure cultures of the fungi isolated even in the heat of summer. On the other hand, some bacterial contamination was observed with the other techniques.

In our procedure, the mycelia penetrate the agar medium without the contaminating bacteria. The fungus uses the mechanical pressure of hyphal tip growth for penetrating the water agar medium. The bacteria can not then pass through the agar pores and the agar medium acts as a sieve for separating the bacteria from the mycelia. Sleeth (1945) used agar mycelium with nutrients in a Petri dish, dividing the medium into quarters and inculating each part in the center. Each part was then put into a separate sterilized Petri dish. The disadvantages of his method are: there is no need to add nutrients to the agar medium since *Pythium* spp. can grow in water agar, and the added nutrients support bacterial growth. He divided the agar medium into quarters and transferred each one to a separate Petri dish, which may allow some of the *Pythium* hypha reach to the surface of the agar block without penetrating it. The technique is laborious and involves the risk of contamination. Robertson (1972, 1980) and De Cock (1986) added 100 μg/ml streptomycin and 100 μg/ml penicillin to water agar to get *Pythium* spp. free from bacteria. Streptomycin at a concentration of 10 μm/ml has been reported to inhibit the growth of *Pythium aphanidermatum* (Edson) Fitz. and *P. irregulare* Buisman (Hine, 1962; McMeekin, 1978). It is advisable to avoid using streptomycin in the selective medium even at low concentrations, because of its suppressive effect upon zoospore production prior to identification in grass blades under aquatic condition, at least in case of *Pythium* spp. (Abdelzaher et al., unpublished data). Streptomycin can interfere with the identification of *Pythium*. The use of successive inoculations in water agar medium (Plaats-Niterink, 1975; Paul and Baghdadi, 1985; Paul, 1986a, b) does not ensure purity, because thin film of bacteria commonly grows along the hyphae. Tsukadaira and Miyata (1981) used a round glass cell, 20 mm diam and 8 mm high, which has a small hole in the center, 2–3 mm diam, and three short legs with a small hole in the bottom of each. In their procedure, the hole in the cell is plugged with loosened glass fiber filter (Whatman GF-A). Melted agar medium is poured to 3–4 mm thick into the sterilized cell kept in a sterilized Petri dish. A sample containing the bacteria-contaminated fungus is put on the agar surface, and cultured for one or two days under suitable con-

Baits kept in pond water at 25 C for 5 days.

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Wash colonized baits thoroughly in sterile distilled water and remove excessive water between sterile filter papers. → Seed these baits on VP_3 medium selective for *Pythium* (Ai-Shtayeh et al., 1986). → Cut a small block of agar medium from the distal end of the colony obtained from the bait, reinoculate it on the center of the surface of 2.5–3.0% water agar medium (7 mm deep* in a 9-cm diam Petri dish) and incubate at 15 C to obtain a colony of about 1 cm diam. → Turn the whole water agar disk upside-down with a flamed forceps under aseptic conditions and then incubate at the same temperature until the colony almost reaches the dish side. Take care to avoid the *Pythium* colony reaching the side of the dish. → Under the microscope, take a thin piece containing a single hyphal tip from the margin of the surface of the *Pythium* colony on water agar and transfer it to a corn meal agar (CMA) slant. The agar piece containing the desired fungus should be as thin as possible. After growing the fungus in the slant culture check for bacterial occurrence by transferring the CMA agar block with the fungus to a tube of nutrient broth (NB) medium and incubating at 28 C for 48 h.

* To secure the best results, the agar medium should be of sufficient thickness and hardness to permit lifting the disk without breaking.

Fig. 1. The recommended technique for isolating bacteria-free *Pythium* spp. from pond water.

ditions. The Petri dish is then opened, and the plug transferred to a new agar plate. The mycelia developed from the plug is transplanted to a slant agar as a stock culture. This technique has some disadvantages in that mycelia can grow along the inner sides of the glass cell without penetrating the agar medium. *Pythium* species tend to grow in contact with a solid surface with the aid of their appressoria (Plaats-Niterink, 1981). This technique is also laborious, and it needs special apparatus. Following the present procedure carefully will give satisfactory results.

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