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# Selective Media for Practical Isolations of *Pythium* spp. From Natural and Agricultural Environments



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## Abstract

Two new *Pythium* selective media without using PCNB and pimaricin are developed. Their practical uses are introduced on isolation of the organs from natural and agricultural environments.

## Introduction

*Pythium* spp. are distributed in wide range of environments in natural and agricultural sites [1]. Selective media is an effective and economic tool for quantitative and qualitative isolations of *Pythium* spp. from plant tissues, soil and water. PARP medium has been the most commonly used for isolation of *Pythium* spp. from soil [2]. The fungicide PCNB is an important component of this medium, but the commercial pesticide products are unavailable since hexachlorobenzene (HCB), which has carcinogenic activity, is present as an impurity in PCNB [3]. Moreover, pimaricin, which is also a necessary component of PARP is not commercially available in several countries including Japan due to the food sanitation law. Therefore efficient alternatives in *Pythium* selective media without using PCNB and pimaricin have been demanded. We previously modified PARP by replacing PCNB in the medium with fluazinam or miconazole, and nystatin with pimaricin [4]. The new media are NARF (nystatin+ampicillin+rifampicin+fluazinam) and NARM (nystatin+ampicillin+rifampicin+miconazole). NARF and NARM are comparable with PARP on yield of naturally occurring *Pythium* species from soil using the soil-dilution plating technique [4]. Since development of NARF and NARM, they were successfully used for isolations of *Pythium* spp. from many locations of natural and agricultural environments [5-7]. This review introduces practical techniques for isolation of *Pythium* spp. from natural and agricultural environments by using these new selective media.

## Media recipe

### Basal medium of NARF and NARM

17g of CMA (Becton Dickinson and Company, Franklin Lakes, NJ, USA)

5g of agar (Wako Pure Chemical Industries, Osaka, Japan) for isolation from plant tissue, or 23g of the agar for using soil-dilution plating

### Antibiotics for NARF

50mg a.i. nystatin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 1ml ethanol

250mg a.i. ampicillin (Sigma-Aldrich) dissolved in 1ml DW

10mg a.i. rifampicin (Sigma-Aldrich) dissolved in 1ml DMSO

0.5mg a.i. of fluazinam (Froncide Wettable Powder®, Nippon Soda, Tokyo, Japan) dissolved in 1ml of 0.1% sterile water agar

### Antibiotics for NARM

10mg/L a.i. nystatin (Sigma-Aldrich) dissolved in 1ml ethanol

250mg/L a.i. ampicillin (Sigma-Aldrich) dissolved in 1ml DW

10mg/L a.i. rifampicin (Sigma-Aldrich) dissolved in 1ml DMSO

1mg/L a.i. miconazole (Sigma-Aldrich) dissolved in 1ml DMSO

Each antibiotics was added to the basal medium after autoclaving and cooling to 50 °C, mixed thoroughly with a magnetic stirrer, and 10ml of medium was poured into 9cm diameter petri plates. If pimaricin is available, 5mg/L a.i. pimaricin can be alternative to nystatin of NARF and NARM.

### ISOLATION FROM PLANT TISSUES

Sections of diseased tissue are washed in tap water, air-dry, and incubate on NARF or NARM usually at 25 °C in darkness. *Pythium* mycelia that grew on the agar are transferred to CMA or water agar.

A Van Tieghem cell method [8] is useful for purifying *Pythium* from bacterial contamination.

### Soil-dilution plating

Approximately one kg of soil corrected from natural and agricultural environments is passed through a 4mm sieve to remove large stones and debris. The 50g of soil is placed in a 500ml flask containing 250ml of autoclaved 0.35% agar. The flask is shaken at 200rpm on a rotary shaker. From each soil dilution flask, a 10ml soil suspension is dispensed into a 500ml Erlenmeyer flask containing 490ml of sterile agar and shaken by hand. A 1ml aliquot of that soil dilution is plated onto each of 10 plates, and a bent glass rod is used to spread the aliquot over the plate. The plates are incubated for 24h in the dark at 25 °C. Following the incubation period, the surface of each plate is gently washed under a stream of water to remove the soil. The plates are further incubated for 24hrs and numbers of colonies of *Pythium* spp. are counted. Colonies of *Pythium* species can be distinguished by colony characteristics such as a fast growing rate, no cross walls in the young mycelium, and wide, highly branched hyphae. Colonies believed to be *Pythium* species are subcultured on CMA and confirm as *Pythium* by microscopic observation of the colonies. The density of *Pythium* spp. is expressed as the number of colonies per g of dry soil.



**Figure 1:** Colonies of *Pythium aphanidermatum* on NARM (nystatin+ampicillin+rifampicin+miconazole agar) in a 9cm Petri dish appeared from bent grass tissues after incubation for 18 h at 31 °C.

### Advantages and disadvantages

NARM is better or equivalent to PARP on *Pythium* growth and inhibition of non-pythiaceous microbes except for *Fusarium* spp. NARM is equivalent to PARP and is significantly better than NARF in having faster growth rates for isolates of *Pythium* (Figure 1). On the other hand, NARM promoted significantly higher growth

of isolates of *Fusarium* spp. as compared to NARF and PARP. This is due to enhancement of miconazole on growth of *Fusarium* spp. in agar culture [9], although the *Fusarium* growth on NARM is still very slow (<2mm/day at 25 °C). NARF can well inhibit growth of *Fusarium* spp. Both media can be used for isolation of *Phytophthora* and *Phytophthium*.

### Toxicity and cost

Both fluazinam [10] and miconazole [11] have low toxicity to humans and the environments. Fluazinam is available as a common commercial fungicide and the cost for the 0.5mg needed to prepare 1 liter of NARF is minimal (<\$0.01). Miconazole is available from Sigma-Aldrich at a cost of \$0.04 for the 1mg needed to prepare 1 liter of NARM. Including costs for the basal medium, the both media require less than \$10 to prepare 1 liter. For their practical uses, CMA (Becton Dickinson and Company) can cost lessly be alternate media such as a handmade cornmeal agar (HCMA) and V8 juice agar (V8JA). Our preliminary results demonstrated that NARF and NARM prepared with HCMA or V8JA are better to them prepared with the commercial CMA on *Pythium* hyphal growth.

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Selective media for practical isolations of *Pythium* spp. from natural and agricultural environments.

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