# Selective Medium for Isolation of *Pythium* spp. from Soil

KENNETH E. CONWAY, Associate Professor, Department of Plant Pathology, Oklahoma State University, Stillwater 74078

**ABSTRACT**

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A selective medium for isolation of *Pythium* spp. from soil and plant tissue was developed by incorporating the broad-spectrum fungicide etaconazole (1.125 EC) into 2% potato-dextrose agar amended with 300 μg/ml of sodium ampicillin. Etaconazole at 17 μg a.i./ml was as effective as a pimaricin-vancomycin-pentachloronitrobenzene medium in recovery of *Pythium arrhenomanes* from artificially infested soil and *Pythium* spp. from wheat, turf, forest nursery, and peanut field soil. Higher concentrations of etaconazole in the medium reduced the recovery of *P. arrhenomanes* and *Pythium* spp. from soil but allowed for mycelial growth in culture.

*Pythium* spp. cause damping-off of many plants and have a worldwide distribution (15). *Pythium* spp. have been associated with economic losses of peanuts (7), wheat (3), and tree seedlings in forest nurseries (18). The incidence and severity of *Pythium* spp. on these crops in Oklahoma have resulted in renewed interest in determining population dynamics of *Pythium* spp. in soil used for their production. Numerous media have been developed for selective isolation of *Pythium* spp. and *Phytophthora* spp. from soil (6,13,14,17). Principles involved in the development of these media include either selective inhibition or enhancement of fungi (16). Selective inhibition is often based on the addition of several antibiotics, fungicides, and other inhibitory chemicals (4,5,9,12). Broad-spectrum fungicides have potential use in developing selective media. One fungicide, etaconazole (Vangard 1.125EC), has a broad spectrum of systemic activity against fungi in the classes Ascomycetes, Basidiomycetes, and Deuteromycetes but has little or no activity against Phycomycetes (1). This research was undertaken to determine if this activity could be incorporated into a selective medium for isolation of species of *Pythium* and/or *Phytophthora* from plant tissue and soil. A preliminary report has been published (2).

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## MATERIALS AND METHODS

**Screening for fungicidal activity.** A basal medium of 2% Difco potato-dextrose agar (PDA) amended with 300 μg/ml of sodium ampicillin was used throughout the experiment. Etaconazole (EANA) (Vangard 1.125EC) (1, 10, 100, and 1,000 μg a.i./ml) was added to the basal medium after autoclaving. Radial growth of *Pythium* sp. from tomato roots, *Rhizoctonia solani* mating groups AG-1, AG-2, and AG-4, *Sclerotium rolfsii* from peanut, *Fusarium moniliforme* from sorghum, *Phytophthora* sp. from *Taxus* sp., *Macrophomina phaseolina* from arborvitae (*Thuja* sp.), *Rhizopus* sp. from soil, and *Trichoderma harzianum* from soil were compared at each concentration. Growth rates were determined at room temperature (25 C) by placing a 1-cm plug from the leading edge of a colony grown on PDA onto the selective medium and measuring the greatest colony diameter every 2 days until most of the cultures reached the edges of the petri dishes. Each treatment was replicated four times, and experiments were conducted at least twice with each organism.

**Comparison of fungal growth.** Excluding *S. rolfsii*, the same fungi used in the screening test were used for this comparison. Growth rates of these fungi on media amended with 100 and 1,000 μg/ml of etaconazole (EANA [100] and EANA [1,000]) were compared with those grown on the modified pimaricin-vancomycin-pentachloronitrobenzene (PVP) medium (17) developed for isolation of *Phytophthora* spp. and *Pythium* spp. from soil. PVP medium consisted of cornmeal agar (17 g/L), pimaricin (10 μg/ml), vancomycin (200 μg/ml), and pentachloronitrobenzene (100 μg/ml). Growth rates were determined as stated for the screening test, except a 0.7-cm inoculum plug was used and measurements were taken at 3, 5, 7, and 10 days. Each treatment was replicated four times.

**Effect of EANA on *Pythium* spp.** Growth of *P. debaryanum, P. splendens, P. ultimum, P. pulchrum, P. vexans, P. mamillatum, P. aphanidermatum, P. irregulare* (obtained from J. McRitchie, Florida Department of Plant Industries, Gainesville), and *P. arrhenomanes* were measured on agar amended with 34 μg/ml of EANA (EANA [34]) to determine if EANA differentially affected species of *Pythium*.

**Selective isolation from soil.** Wheat cultivar TAM W 101 was grown in an unsterilized field soil artificially inoculated with mycelium of *P. arrhenomanes*. After 2 mo, the soil was air-dried, sieved (2-mm screen), and stored in a loosely covered bucket at room temperature.

Soil samples were collected from fields in different locations in Oklahoma that had been planted to turf (Stillwater), peanut (Perkins), forest nursery (Washington), and wheat (Stillwater). Each soil was thoroughly mixed, air-dried, and sieved before analysis.

Media used included PVP and PDA amended with 300 μg/ml of sodium ampicillin and two concentrations of EANA (17 and 34 μg/ml). Two grams of soil from each location were added to 38 ml of a dilution suspension containing water agar (0.3%), $CaCl_2 \cdot 2H_2O$ (0.368 g/100 ml) and adjusted to pH 5.5 with 1% phosphoric acid. This suspension was mixed and 1 ml was spread on the selective medium in each of 10 petri dishes. After 24 hr of incubation at 25 C in the dark, the surface of agar in each petri dish was gently washed in a stream of water to remove soil and bacterial growth. Colonies of *Pythium* spp. were counted after additional incubation of 24, 48, 72, and 96 hr. The experiment was performed twice with infested soil and three times with each field soil. Representative colonies were transferred to PDA for verification and speciation of *Pythium*. Average number of *Pythium* colonies from 10 replicated petri dishes for each medium were analyzed using a one-tailed *t* test with the null hypothesis stated to ascertain if $\bar{X}_{PVP} \leq \bar{X}_{EANA}$.

## RESULTS AND DISCUSSION

**Screening for fungicidal activity.** Greatest growth inhibition on the media was obtained when both EANA and sodium ampicillin were added to PDA after autoclaving. First evaluations of inhibition were made 2 days after inoculation. Concentrations of 1 and 10 μg/ml of EANA were not sufficient to

differentiate *Rhizopus* sp. and *R. solani* AG-4 from *Pythium* sp. Colony diameters of fungi grown on media amended with 100 and 1,000 μg/ml of EANA are shown in Table 1. After 2 days of incubation, EANA (100) inhibited growth of all fungi except *Pythium* sp., which covered the entire surface of the medium in the petri dishes. After 4 days of incubation, *Rhizopus* sp. had also overgrown the medium. EANA (100) inhibited growth of *R. solani* AG-1, *S. rolfsii, F. moniliforme,* and *T. harzianum* for up to 10 days of incubation.

EANA (1,000) gave excellent inhibition of all fungi except *Pythium* sp. for up to 10 days. *R. solani* AG-1, *S. rolfsii, F. moniliforme, Rhizopus* sp., and *T. harzianum* were inhibited for up to 30 days.

**Comparison of fungal growth.** After 3 days of incubation, *Pythium* sp. grew significantly more on EANA (1,000) and PVP than the other fungi (Table 2). Growth of *Pythium* sp. and *Rhizopus* sp. was similar on EANA (100) and was greater than that of other fungi. *Pythium* sp. and *Phytophthora* sp. were inhibited more on EANA (1,000) than on EANA (100) or PVP.

After 10 days of incubation on EANA (100), *Pythium* sp., *R. solani* AG-4, *Rhizopus* sp., and *M. phaseolina* had all completely covered the agar surface. On PVP, *Pythium* sp., *R. solani* AG-4, and *T. harzianum* also covered the agar surface. However, only *Pythium* sp. completely covered the agar surface of EANA (1,000) because the other fungi were still being inhibited.

**Effect of EANA on *Pythium* spp.** Seven of the nine *Pythium* isolates completely covered the amended agar (EANA [34]) surface (85 mm) by the third day. *P. pulchrum* and *P. vexans* did not reach full diameter until after the fifth day on both EANA (34) and PDA. There were no indications that EANA (34) inhibited mycelial growth of *Pythium* spp. tested. *Pythium* spp. in this experiment grew as rapidly as *Pythium* sp. from tomato grown on agar amended with EANA (100).

**Selective isolation from soil.** Although many selective media have been developed to isolate *Pythium* spp. from soil, PVP medium has been used preferentially. EANA (100) has been used routinely in our Plant Disease Diagnostic Laboratory (2) as an easy-to-prepare and inexpensive medium for isolation of *Pythium* spp. and *Phytophthora* spp. from diseased plant tissue. However, initial comparisons of isolation efficacy from soil indicated that 100 μg/ml of EANA was too inhibitory to *Pythium* spp. to allow for comparison with PVP. Concentrations of 17 and 34 μg/ml of EANA were incorporated into a basal medium and used for comparison with PVP (Table 3). Comparison of propagule counts from the selective media indicated that *Pythium* spp. were recovered on PVP

**Table 1.** Growth (cm) of selected fungi on a medium amended with etaconazole at two concentrations

| Fungus | EANA (100)[a] (days of growth) | | | EANA (1,000)[a] (days of growth) | | | |
|---|---|---|---|---|---|---|---|
| | 2 | 4 | 10 | 2 | 4 | 10 | 30 |
| *Pythium* sp. | 8.2[b] | 8.5 | 8.5 | 3.8 | 7.9 | 8.5 | 8.5 |
| *Phytophthora* sp. | 2.1 | 3.5 | 7.6 | 1.3 | 1.9 | 4.3 | 7.8 |
| *Rhizoctonia solani* | | | | | | | |
| AG-1 | 1.6 | 2.3 | 2.3 | 1.2 | 1.3 | 1.3 | 1.3 |
| AG-2 | 1.6 | 2.8 | 6.2 | 1.1 | 1.8 | 2.8 | 8.0 |
| AG-3 | 2.0 | 3.2 | 7.8 | 1.4 | 2.5 | 4.5 | 8.5 |
| *Rhizopus* sp. | 3.8 | 8.5 | 8.5 | 1.0 | 1.0 | 1.0 | 1.0 |
| *Trichoderma harzianum* | 1.0[c] | 1.5 | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| *Macrophomina phaseolina* | 1.7 | 4.0 | 8.5 | 1.2 | 2.4 | 3.9 | 8.2 |
| *Fusarium moniliforme* | 1.0 | 1.2 | 2.0 | 1.0 | 1.0 | 1.4 | 4.5 |
| *Sclerotium rolfsii* | 1.5 | 1.8 | 2.8 | 1.0 | 1.1 | 1.2 | 1.7 |

[a] Basal medium consisted of 2% potato-dextrose agar and 300 μg/ml of sodium ampicillin with the addition of either 100 or 1,000 μg/ml of etaconazole (EANA).
[b] Each value is the average of the greatest diameter of each colony from two trials with four observations per trial.
[c] Values of 1.0 indicate no growth from the plug used to inoculate the media.

**Table 2.** Growth (cm) of selected fungi on three selective media 3 and 10 days after inoculation

| Fungus | Days after inoculation: 3 | | | 10 | | |
|---|---|---|---|---|---|---|
| | EANA (100)[a] | EANA (1,000)[a] | PVP[b] | EANA (100) | EANA (1,000) | PVP |
| *Pythium* sp. | 8.5[c] | 4.0 | 8.5 | 8.5 | 8.5 | 8.5 |
| *Phytophthora* sp. | 2.3 | 0.8 | 4.3 | 6.7 | 1.5 | 8.5 |
| *Rhizoctonia solani* | | | | | | |
| AG-1 | 2.2 | 1.0 | 1.1 | 3.3 | 1.3 | 5.0 |
| AG-2 | 1.8 | 0.8 | 1.0 | 2.9 | 1.2 | 4.0 |
| AG-4 | 3.6 | 1.6 | 1.6 | 8.5 | 3.8 | 8.3 |
| *Rhizopus* sp. | 8.3 | 0.9 | 0.7[d] | 8.5 | 1.0 | 0.7 |
| *Trichoderma harzianum* | 1.9 | 0.7 | 2.5 | 4.5 | 0.7 | 8.5 |
| *Macrophomina phaseolina* | 2.6 | 1.9 | 1.2 | 8.5 | 4.9 | 4.1 |
| *Fusarium moniliforme* | 0.9 | 0.7 | 1.3 | 2.0 | 0.8 | 4.8 |

[a] Basal medium consisted of 2% potato-dextrose agar and 300 μg/ml of sodium ampicillin with the addition of either 100 or 1,000 μg/ml of etaconazole (EANA).
[b] PVP medium consisted of cornmeal agar (17 g/L), pimaricin (10 μg/ml), vancomycin (200 μg/ml), and pentachloronitrobenzene (100 μg/ml).
[c] Each value is an average of the greatest diameter of each colony from four observations.
[d] Values of 0.7 indicate no growth from the plug used to inoculate the media.

**Table 3.** Enumeration of *Pythium* spp. (propagules/g of soil) from various fields on three selective media

| Medium | Source of soil for analysis: Artificially infested wheat[a] | | Wheat | | | Peanut | | | Forest | | | Turf | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1[b] | 2 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| PVP[c] | 70 | 117 | 174 | 202 | 182 | 74 | 78 | 70 | 6 | 16 | 16 | 6 | 10 | 4 |
| EANA (17)[d] | 126 | 120 | 176 | 156[e] | 116[e] | 46 | 80 | 48 | 18 | 8 | 2 | 4 | 12 | 8 |
| EANA (34)[d] | 82 | 102 | ...[f] | 4[e] | 4[e] | 6[e] | 18[e] | 10[e] | 6 | 6 | ... | ... | ... | ... |

[a] Soil was collected from a wheat field and artificially inoculated with *Pythium arrhenomanes*.
[b] Each column represents a trial, and each value is a mean of colonies per dish (multiplied by dilution factor of 20), 10 petri dishes per trial.
[c] PVP medium consisted of cornmeal agar (17 g/L), pimaracin (10 μg/ml), vancomycin (200 μg/ml), and pentachloronitrobenzene (100 μg/ml).
[d] Basal medium consisted of 2% potato-dextrose agar and 300 μg/ml of sodium ampicillin with the addition of either 17 or 34 μg/ml of etaconazole (EANA).
[e] Values are significantly less ($P = 0.05$) than with PVP medium as determined by a one-tailed $t$ test.
[f] No colonies of *Pythium* spp. recovered.

medium in significantly greater numbers in only two of 14 trials compared with EANA (17). The concentration of etaconazole in EANA (34) was too inhibitory for comparable recovery of *Pythium* spp. from soil. Most cultures isolated from each medium belonged to the *P. debaryanum-P. irregulare* complex (8). In addition, *P. ultimum* was recovered on PVP from forest soil. Other *Pythium* spp. recovered on EANA (17) included *P. ultimum* from peanut soil and *P. papillatum* and *P. torulosum* from turf. It is interesting to note that most isolates from each medium possessed sphaerical sporangia. Neither *P. myriotylum* from peanut nor *P. arrhenomanes* from wheat were recovered on selective media. *P. myriotylum* was isolated from peanut tissue from plants in the same field (H. A. Melouk, *personal communication*); however, difficulty in recovery of *P. myriotylum* from soil has been reported previously (10). *P. arrhenomanes* infects wheat seedlings during late fall and winter and may not have been active when soil samples were collected from the fallow wheat field in September when soil temperatures were 30–40 C. Contamination by Mucoraceous fungi was not a problem with EANA or PVP media. Bacterial contamination was not as great on EANA media as on PVP, which permitted easier isolation of clean cultures of *Pythium* spp. from EANA.

The greatest benefit of using EANA (17) or EANA (34) was the length of time that *Pythium* spp. were restricted and countable. PVP medium was rapidly overgrown by *Pythium* spp. and had to be counted within 48 hr of incubation. EANA (17) and EANA (34) produced discrete colonies and were best counted between 60 and 72 hr and 72 and 96 hr, respectively.

Mircetich and Kraft (11) noted a differential sensitivity between mycelia and spores of *Pythium* spp. to pimaricin. Greater recovery of *Pythium* spp. was accomplished using lower concentrations of pimaricin. A differential sensitivity for EANA was also noted in recovery of *Pythium* spp. from soil. Concentrations of EANA that were effective in recovery of *Pythium* spp. from plant tissue were too great for adequate recovery from soil. EANA (17) was as good as PVP for recovery of *Pythium* spp. from soil in 12 of 14 trials, allowed a longer period for counting of colonies, recovered a similar spectrum of *Pythium* spp. as PVP medium, and is less expensive to prepare.


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