Selective Inhibition of Pythium spp. on a Medium for Direct Isolation of Phytophthora spp. from Soils and Plants

H. Masago, M. Yoshikawa, M. Fukada, and N. Nakanishi

Professor, Assistant Professor, and Undergraduate Students, respectively, Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto Prefectural University, Shimogamo, Kyoto 606, Japan.

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ABSTRACT

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The growth of *Pythium* spp., but not *Phytophthora* spp., was selectively inhibited by 3-hydroxy-5-methylisoxazole (HMI). Incorporation of HMI at 25 and 50 μg/ml into a potato-dextrose agar medium containing other antimicrobial agents (benomyl, nystatin, pentachloronitrobenzene, rifampicin, and ampicillin at 10, 25, 25, 10, and 500 μg/ml, respectively) almost completely prevented colony development of bacteria, nonpythiaceous fungi, and 12 species of *Pythium*, but did not significantly affect mycelial growth and spore germination of various *Phytophthora* spp. With this selective medium, the presence and propagule density of *Phytophthora* spp. in soil could be quantitatively determined. The medium also was useful for isolating *Phytophthora* spp. from plant tissues.

Additional key words: selective medium, selective antimicrobial chemical, differential toxicity, phycomycetes, pathogen population.

Selective media for the direct isolation of *Phytophthora* spp. from soils and plant tissues have been devised that contain various antimicrobial agents (4). A major shortcoming of those media, however, appears to be that none will select for *Phytophthora* spp. and inhibit *Pythium* spp. Since the growth of *Pythium* spp. usually is much more rapid than that of *Phytophthora* spp., pure and quantitative isolation of *Phytophthora* spp. becomes difficult if the samples such as soil are heavily contaminated by *Pythium* spp. Our objective, therefore, was to develop a selective medium for *Phytophthora* spp. that would prevent development of *Pythium* spp., other fungi, and bacteria, and permit direct isolation of *Phytophthora* spp. from soils and plants. We found that 3-hydroxy-5-methylisoxazole (HMI) selectively inhibited *Pythium* spp. with little or no adverse effects upon *Phytophthora* spp.; a medium supplemented with HMI and other antimicrobial agents therefore almost completely fulfilled our experimental purpose.

MATERIALS AND METHODS

For the preparation of the selective medium, potato-dextrose agar (PDA, 1% agar) was supplemented with the following antifungal agents: benomyl (Benlate, 50% active; E. I. DuPont de Nemours, Wilmington, DE 19898), nystatin (Mycostatin 2,000 units/mg; Serva, Heidelberg, Germany), and PCNB (pentachloronitrobenzene); and with the following antibacterial agents: rifampicin (Rifampin; Calbiochem, San Diego, CA 92112) and ampicillin (Viccillin; Meiji Seika, Tokyo, Japan); at 10, 25, 25, 10, and 500 μg/ml, respectively (henceforth called the BNPRA medium). The compound, 3-hydroxy-5-methylisoxazole (Tachigaren; Sankyo, Tokyo, Japan; hereafter referred to as HMI) or its derivatives (kindly supplied by Sankyo) was added to the BNPRA medium at various concentrations. All concentrations were based on active ingredients. Each antimicrobial agent was first dissolved or finely suspended separately in 80% ethanol as concentrated stocks, which were mixed at appropriate proportion and then diluted with sterile distilled water to give a 10-fold concentration of that in the final medium on the day of use. One milliliter each of the diluted chemical solution and soil suspension was placed into a 9-cm diameter petri plate. Care was taken to avoid contact between the chemical solution and soil suspension before adding 8 ml of melted PDA (at about 45 C) and mixing. The plates were incubated at 28 C. The final ethanol concentration in the medium was 0.5% or less, which was nontoxic to all organisms studied.

Germination of *Phytophthora* spores was studied in potato-dextrose broth (PDB) that contained the antimicrobial agents at the same concentrations as those in the selective medium. Oospores of *P. castaneae* Katsura et Uchida were harvested from 14-day-old cultures in liquid V-8 juice medium (10% V-8 juice, 0.2% $CaCO_3$) by homogenizing the mycelia with 100 ml of deionized water in a Waring Blendor for 2 min at full

speed. Then oospores were separated from the mycelium by washing and centrifugation (approximately 500 *g*) three to five times. Chlamydospores of *P. parasitica* Dastur were prepared by the method of Tsao (5) and zoosporangia and zoospores of *P. capsici* Leonian by the method of Katsura et al. (2). Encysted zoospores (cysts) were obtained by vigorously shaking zoospores in deionized water. Two-tenths milliliter of each spore suspension was mixed with 3 ml of PDB and incubated at 28 C in the light. Rates of spore germination were recorded after 6 hr for zoosporangia and cysts, and after 24 hr for oospores and chlamydospores.

RESULTS

The HMI and derivatives such as 3-hydroxy-5-ethyl- and 3-hydroxy-5-*n*-propylisoxazoles possessed marked differential toxicity toward *Phytophthora* and *Pythium* spp. at 25 and 50 μg/ml (Table 1). Although the selective toxicity exerted by some of the derivatives was comparable to that by the parent compound, HMI was chosen as the agent to be incorporated into the selective medium for *Phytophthora* because of its commercial availability.

The BNPRA medium is known to selectively favor the growth of pythiaceous fungi (Masago et al., *unpublished*), and the addition of 25 or 50 μg/ml of HMI increased its selectivity by inhibiting *Pythium* spp. but permitting growth of *Phytophthora* spp. (Table 2). Although some *Pythium* spp. grew slightly on the BNPRA-HMI medium with very thin colony appearance, we have not isolated *Phytophthora* spp. from soils or plant tissues that were contaminated with *Pythium*. The BNPRA-HMI medium, furthermore, did not inhibit germination of various *Phytophthora* spores, including oospores of *P. castaneae,* chlamydospores of *P. parasitica,* and zoosporangia and cysts of *P. capsici* (Table 3).

Pythium spp. interfered with the isolation of *Phytophthora melonis* Katsura from naturally infected cucumber roots and their rhizosphere soil on the BNPRA medium (Fig. 1), but the same medium supplemented with HMI yielded no recognizable *Pythium* colonies. In addition, the BNPRA-HMI medium may be useful for

TABLE 1. Effect of 3-hydroxy-5-methylisoxazole and its derivatives on linear growth of certain *Phytophthora* and *Pythium* spp. on potato-dextrose agar medium

Chemical	Chemical in agar (μg/ml)	Colony radius (mm) at 2 days[a]			
		Phytophthora		*Pythium*	
		capsici	*palmivora*	*aphanidermatum*	*debaryanum*
None	0	14	15	>42	29
3-Hydroxy-5-methylisoxazole (HMI)	12.5	14	14	28	2
	25.0	13	12	5	3
	50.0	15	13	3	3
	100.0	10	12	1	0
3-Hydroxy-5-ethylisoxazole	12.5	14	13	10	10
	25.0	14	12	4	8
	50.0	11	10	0	3
	100.0	9	7	0	0
3-Hydroxy-5-*n*-propylisoxazole	12.5	14	13	27	15
	25.0	14	12	6	1
	50.0	10	6	0	3
	100.0	5	4	0	0
3-Hydroxy-5-phenylisoxazole	12.5	12	8	33	3
	25.0	0	0	3	0
	50.0	0	0	0	0
	100.0	0	0	0	0
3-(*p*-toluoyloxy)-5-methylisoxazole	12.5	13	12	>42	13
	25.0	14	13	>42	16
	50.0	13	15	>42	13
	100.0	12	12	35	3
3-(*o*-toluoyloxy)-5-methylisoxazole	12.5	15	12	34	7
	25.0	13	12	25	4
	50.0	13	9	5	0
	100.0	5	5	1	0
5-Methyl-N-carbo-ethoxy-3-isoxazolone	12.5	12	8	32	2
	25.0	5	3	24	1
	50.0	3	2	10	1
	100.0	0	1	0	0

[a]Readings were averages of two replicate plates.

TABLE 2. Growth of different fungi on potato-dextrose agar medium (control) and on the same medium containing benomyl, nystatin, PCNB, rifampicin, and ampicillin (BNPRA) at 10, 25, 25, 10, and 500 μg/ml, respectively, and 3-hydroxy-5-methyl-isoxazole (HMI) at the specified concentrations

	Colony radius (mm) at 2 days[a]				
		HMI	BNPRA + HMI (μg/ml)		
Organism	Control	50 μg/ml	0	25	50
Phytophthora cactorum (Leb. et Cohn) Schroet.	6	5	5	4	3
P. capsici Leonian	13	13	11	10	11
P. castaneae Katsura et Uchida	12	11	9	9	8
P. cinnamomi Rands	11	11	8	10	10
P. citricola Sawada	9	8	5	7	5
P. cryptogea Pethyb. et Laff.	7	7	6	7	6
P. drechsleri Tucker	11	11	6	6	7
P. erythroseptica Pethyb.	13	11	8	7	8
P. melonis Katsura	9	10	8	8	7
P. parasitica Dast.	8	8	7	8	9
P. palmivora (Butler) Butler	13	12	11	9	10
P. phaseoli Thaxt.	19	16	17	18	18
Pythium aphanidermatum (Edson) Fitzp.	>42	5	>42	7	2
P. betae Takahashi	18	3	5	0	0
P. debaryanum Hesse	27	0	18	0	0
P. irregulare Buisman	>42	7	>42	9	4
P. mamillatum Meurs	>42	6	>42	8	5
P. monospermum Pringsheim	30	4	21	2	2
P. nelumbium Tak.	>42	8	20	7	3
P. ostracodes Drechs.	>42	2	22	1	1
P. spinosum Saw. spud Saw. et Chen	18	0	19	0	0
*P. ultimum*Trow	>42	2	35	0	1
P. vexans dBy	>42	0	23	2	0
P. sp.	>42	0	>42	2	1
Alternaria radicina Meier, Drechs. et Eddy	10	...[b]	0	0	0
Ascochyta pisi Lib.	4	...	0	0	0
Aspergillus niger van Tiegh.	9	...	0	0	0
Botrytis cinerea Pers. ex Fr.	6	...	0	0	0
Chaetomium sp.	5	...	0	0	0
Colletotrichum lagenarium (Pass.) Ellis & Halst.	12	...	0	0	0
Fusarium sp.	13	...	0	0	0
Penicillium notatum Westl.	15	...	0	0	0
Rhizoctonia solani Kühn	25	...	0	0	0
Rhizopus nigricans Ehrenb.	>42	...	4	3	3
Sclerotinia sp.	4	...	0	0	0
Trichoderma sp.	21	...	1	1	1

[a]Readings were averages of two replicate plates.
[b]Not evaluated.

the quantitative enumeration of propagule populations of *Phytophthora* spp. in soils, since the recovery from artificially-infested soil was high (Table 4). The composition of the selective medium, now routinely used in this laboratory for the isolation of *Phytophthora* spp. from soils or infected plants, is: PDA (1% agar) supplemented with benomyl, nystatin, PCNB, rifampicin, ampicillin, and HMI at 10, 25, 25, 10, 500, and 25 or 50 μg/ml, respectively.

DISCUSSION

The HMI and some of its derivatives have been shown to be effective for controlling certain soilborne plant pathogens including *Pythium* spp., but they have not been observed to control *Phytophthora* spp. (1, 3). Our

TABLE 3. Germination of various types of spores of *Phytophthora* spp. in potato-dextrose broth medium (control) and on the same medium containing benomyl, nystatin, PCNB, rifampicin, and ampicillin (BNPRA) at 10, 25, 25, 10, and 500 μg/ml, respectively, and 3-hydroxy-5-methylisoxazole (HMI) at the specified concentrations

	Germination			
		BNPRA + HMI (μg/ml)		
Spore[a]	Control (%)	0 (%)	25 (%)	50 (%)
Zoosporangium	92	99	94	96
Cyst	48	48	41	39
Chlamydospore	20	20	14	16
Oospore	15	16	22	15

[a]Zoosporangia and cysts of *P. capsici*, chlamydospores of *P. parasitica*, and oospores of *P. castaneae* were tested.

TABLE 4. Recovery of *Phytophthora* spp. from a field soil supplemented with different *Phytophthora* spores on potato-dextrose agar medium (control) and on the same medium containing benomyl, nystatin, PCNB, rifampicin, and ampicillin (BNPRA) at 10, 25, 25, 10, and 500 μg/ml, respectively, and 3-hydroxy-5-methylisoxazole (HMI) at the specified concentrations

Spore type introduced into soil[a]	Calculated no. of spores introduced per plate	*Phytophthora* colonies recovered[b]			
		Control	BNPRA + HMI (μg/ml)		
			0 (no.)	25 (no.)	50 (no.)
Zoosporangium	25	0	21[c]	15	18
Cyst	50	0	29[c]	30	27
Chlamydospore	100	0	11[c]	13	17
Oospore	100	0	12[c]	10	6

[a]Soil was diluted to 1/200 by sterile water and then the soil suspension was mixed with the appropriate numbers of each spore. The sources of various spores were the same as Table 3.

[b]Readings were averages of three replicate plates at 3 days and figures are expressed as an average per plate.

[c]*Pythium* spp. overgrew these plates and counting of the *Phytophthora* colonies could not be made on some of the plates where *Pythium* spp. were present.

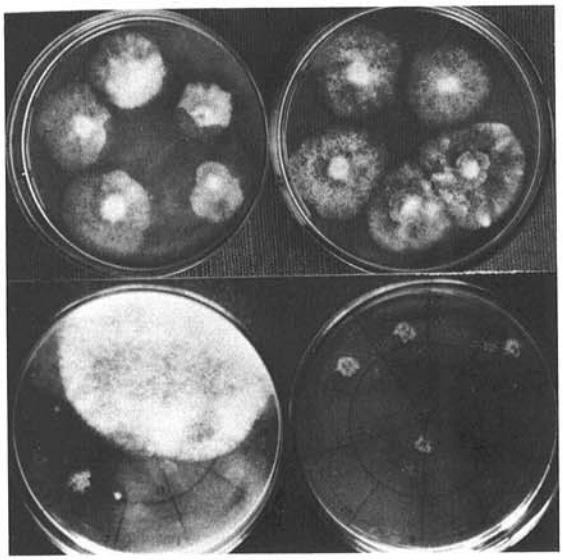

Fig. 1. Isolation of *Phytophthora melonis* from cucumber roots and associated rhizosphere soil. The two plates at the left contained benomyl, nystatin, PCNB, rifampicin, and ampicillin at 10, 25, 25, 10, and 500 μg/ml, respectively (BNPRA), and the two at the right the same ingredients but supplemented with 3-hydroxy-5-methylisoxazole at 25 μgPml (BNPRA-HMI). Upper and lower plates were incubated for 4 and 2 days, respectively. Note that only *P. melonis* colonies are apparent on the two plates at right and that the two plates at left are contaminated by *Pythium* spp., including sparse colony that completely covered the upper plate and a distinctive colony on one half of the lower plate.

results indicated that this is due to selective toxicity, since HMI and derivatives such as 3-hydroxy-5-ethyl- and 3-hydroxy-5-*n*-propylisoxazoles possessed marked differential toxicity toward *Phytophthora* and *Pythium* species.

A serious difficulty in the use of selective media for isolation of *Phytophthora* spp. from soil is overgrowth of plates by *Pythium* spp. Incorporation of HMI into the BNPRA medium improved the selectivity of the medium so that *Phytophthora* spp. were readily recovered from soils and plant tissues without contamination by *Pythium* spp. Although the inhibition of *Pythium* spp. by HMI in vitro was not complete and permitted slight growth of *Pythium* spp., threre was no instance in which colonies of *Pythium* spp. developed on soil dilution-plates that contained HMI.

The BNPRA medium supplemented with HMI did not interfere with mycelial growth by *Phytophthora* spp., nor did this medium inhibit germination and colony establishment of oospores and chlamydospores, the likely forms of the fungus existing in soils (4). This, and the fact that with the HMI medium *Phytophthora* spp. could be quantitatively recovered when added to field soils (Table 4), indicated that the medium should be useful for determining propagule densities of the fungus in soils.

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