Inhibition of Mortierella and Pythium in a Phytophthora-isolation Medium Containing Hymexazol

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ABSTRACT

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Hymexazol (3-hydroxy-5-methylisoxazole, or HMI) at 50 μg/ml, which is noninhibitory to most *Phytophthora* spp., not only inhibited the development of *Pythium* as reported previously by Masago et al., but also *Mortierella* spp. which often are present in high populations in soil and can interfere with the detection and enumeration of *Phytophthora* in soil dilution plates. The pimaricin-vancomycin-pentachloronitrobenzene medium supplemented with hymexazol at 50 μg/ml (PVPH medium) was effective for selective isolation and quantitative estimation of *Phytophthora cinnamomi* and *P. parasitica* from roots and soils. Results of in vitro tests showed that linear growth of all 11 *Mortierella* isolates, but not all 10 *Pythium* isolates, was greatly or completely inhibited on PVPH medium. Some isolates of *Phytophthora cactorum* and *P. palmivora* were more sensitive to hymexazol than *P. cinnamomi*, *P. citrophthora*, *P. parasitica*, and the atypical black pepper strain of '*P. palmivora*'. Hymexazol reduced colony density of most test fungi, including some *Phytophthora* spp.

Additional key words: selective medium, differential medium, selective inhibition.

A number of selective agar media containing antibacterial antibiotics and selective antifungal agents such as nystatin, pimaricin, pentachloronitrobenzene (PCNB) and/or benomyl (1, 2, 8, 13, 14) have proved useful for isolating various *Phytophthora* spp. from soil and plant tissues. All these media, however, also allow the growth of *Pythium* spp. and most *Mortierella* spp. (13, and Tsao and Guy, *unpublished*) which are often present in soils and infected tissues. When present in high populations, these undesired nontarget fungi obscure the detection of *Phytophthora* on selective media or make quantitative estimation of soil populations of *Phytophthora* difficult or inaccurate (9, 13). An efficient chemical agent capable of selectively inhibiting or minimizing the development of *Pythium* and *Mortierella* spp. has been sought by those working with *Phytophthora* spp. for many years without success.

Masago et al. (5) recently reported that 3-hydroxy-5-methylisoxazole (HMI, or hymexazol) at 50 μg/ml or lower concentrations completely or greatly inhibited the growth of 12 *Pythium* spp., but was virtually noninhibitory to 12 *Phytophthora* spp. that were tested. It was also noninhibitory to germination of various kinds of spores of selected *Phytophthora* spp. They devised a selective medium by incorporating hymexazol (HMI in their paper) at 25 or 50 μg/ml into a potato-dextrose agar medium containing benomyl, nystatin, PCNB, rifampicin, and ampicillin (BNPRA medium) (2) and reported that the new BNPRA + HMI medium allowed easy detection and recovery of *Phytophthora melonis* from infected cucumber roots and from a cucumber rhizosphere soil without interference from associated *Pythium* spp. (5).

By incorporating hymexazol at 50 μg/ml in our standard *Phytophthora*-isolation medium, $P_{10}VP$ (14), we have confirmed their results on *Pythium* inhibition and improved *Phytophthora* recovery from various nonsterile soils. An additional observation from the results of our tests has been the complete inhibition of *Mortierella* spp. in soil dilution plates containing hymexazol. The purpose of this paper is to supplement as well as confirm the remarkable discovery of Masago et al. (5), and to report the hitherto unnoticed phenomenon of *Mortierella* inhibition by hymexazol in the selective medium.

MATERIALS AND METHODS

The hymexazol [3-hydroxy-5-methylisoxazole, or HMI (5); trade name Tachigaren] used was manufactured by Sankyo Co., Yasu, Shiga-ken, Japan (12). Samples used in our tests were water-soluble white powders having purities of 98.9 or 99.4%. This relatively new soil fungicide and its several derivatives are known to be effective against various pathogenic fungi, including

Pythium spp., at relatively low concentrations, but are noninhibitory at 1,000 μg/ml against certain bacteria, yeasts, and several *Phytophthora* spp. that have been tested (3, 10, 12). There are other reports on its chemistry, mechanism of toxicity, function-structure relationships, toxicology, metabolism, and resistance (4, 6, 7, 11, 12).

The basal medium for testing selective toxicity of hymexazol was the pimaricin-vancomycin-PCNB ($P_{10}VP$) medium (14). The medium contains, per liter of water, 17 g Difco cornmeal agar, 10 mg pimaricin (Pimafucin, 90.5%, Gist-Brocades N.V., Delft, Holland), 200 mg vancomycin (Vancocin, 100%, Eli Lilly and Co., Indianapolis, IN 46206) and 100 mg PCNB (Terraclor, 75% wettable powder, Olin Mathieson Chemical Corp., Little Rock, AR 72203), all concentrations being based on active ingredients. The procedure for medium preparation was the same as reported previously (14). Hymexazol, together with other antimicrobial agents, was added to the medium as a concentrated (10-fold) stock solution before the plates were poured. In addition to the $P_{10}VP$ medium, Difco cornmeal agar medium was used as the basal medium in some in vitro sensitivity tests.

Numbers of recoverable propagules of *Phytophthora* in soils were compared on the $P_{10}VP$ medium with or without hymexazol at 50 μg/ml by means of the soil dilution plate method. Soils used were nonsterile field

TABLE 1. Recovery of *Phytophthora cinnamomi* colonies and undesired colonies of *Pythium* and *Mortierella* spp. in soil dilution plates on the pimaricin-vancomycin-PCNB ($P_{10}VP$) medium with and without 50 μg/ml hymexazol

			Colonies per plate (avg. no.)					
			Phytophthora cinnamomi		*Pythium* spp.		*Mortierella* spp.	
			[Hymexazol concn. (μg/ml) in $P_{10}VP$ medium]					
Test	Soil[a]	Dilution	0	50	0	50	0	50
1	1	1/30	3.9[b]	4.7	0.5	0	352	0
	2	1/30	13.1	15.9	0.2	0	144	0
2	3	1/100	10.6	9.7	0.1	0	>100	0
3	4	1/50	5.7	8.9	0.3	0	>150	0
4	5[c]	1/30	7.2	9.8	0.5	0	187	0

[a]Nonsterile avocado field soils involved in experiments on soil amendments were used, each artificially infested with chlamydospores of *Phytophthora cinnamomi* at 5, 12, or 19 days prior to soil dilution-plate tests.

[b]Results read at 2 or 3 days after plating; average of 10 replicate plates per medium. Numbers of *P. cinnamomi* colonies are not significantly different ($P = 0.05$) between 0 and 50 μg/ml hymexazol in any tests. Detection of *Phytophthora* colonies was more difficult on the medium without hymexazol, however; accurate counting beginning at 24-26 hr often was necessary.

[c]The $P_{10}VP$ medium containing 25 μg/ml hymexazol was also used in the test with this soil. Average numbers of colonies of *Phytophthora*, *Pythium*, and *Mortierella* recovered on this medium were eight , zero, and five per plate, respectively.

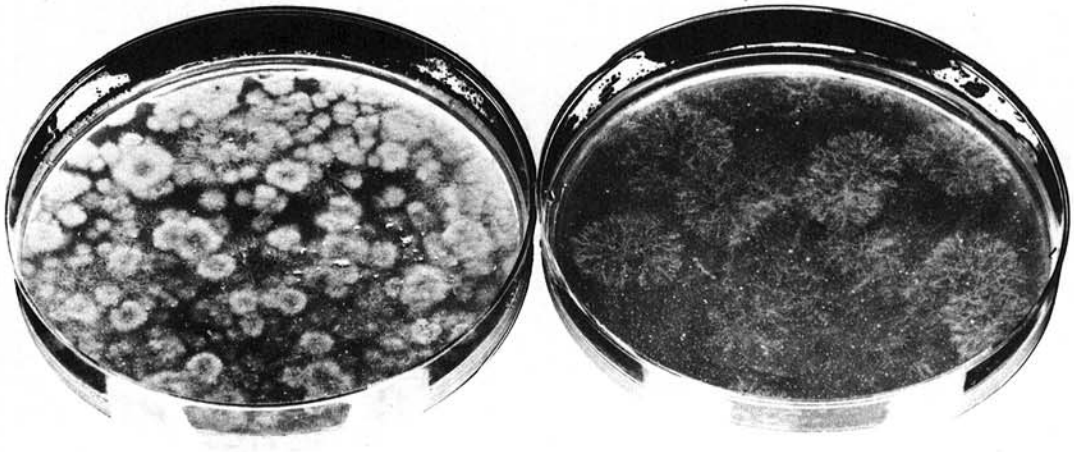

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Fig. 1. Comparison of soil dilution plates containing 1 ml of a 1 in 30 dilution (or 33 mg) of a nonsterile, *Mortierella*-laden, avocado soil artificially infested with chlamydospores of *Phytophthora cinnamomi* 19 days before the soil was assayed. The plates were photographed 2 days after plating. **Left**, the pimaricin-vancomycin-PCNB ($P_{10}VP$) medium showing more than 150 colonies of *Mortierella* spp. (smaller, dense colonies), one *Pythium* colony (nondiscernible in the figure), and some *P. cinnamomi* colonies (larger and more sparse); **right**, the same medium containing 50 μg/ml hymexazol showing complete inhibition of development of *Mortierella* and *Pythium* spp. with almost a pure stand of *P. cinnamomi* colonies. Some bacterial colonies were seen in both media.

soils collected from avocado or citrus groves either naturally infested with *Phytophthora cinnamomi* Rands or *P. parasitica* Dast. [*P. nicotianae* var. *parasitica* (Dast.) Waterh.], or artificially infested with chlamydospores of these respective fungi. Some soils used were those involved in organic amendment experiments and were extremely high in *Mortierella* populations. Soil dilutions of 1 in 30, 1 in 50, or 1 in 100 were used and procedures were similar to those reported previously (14). Ten to 12 replicate plates per medium were used for each soil sample, and *Phytophthora* colony counts were made at 24-26 hr and again at 48-52 hr; in some experiments a reading also was made at 72-76 hr. Numbers of *Pythium* and *Mortierella* colonies that developed on each medium also were recorded. With certain naturally infested soils containing low *Phytophthora* populations, direct inoculation method (by sprinkling about 100 mg of soil evenly on the surface of the agar medium) also was employed; 10-20 replicate plates were used for each soil with each medium. From some soils avocado roots naturally infected with *P. cinnamomi* were plated out, without surface sterilization, on $P_{10}VP$ medium containing 0 or 50 μg/ml hymexazol.

The in vitro effects of hymexazol on growth were tested on 13 isolates of *Phytophthora*, 10 isolates of *Pythium*, and 11 isolates of *Mortierella* (see Table 2). In most tests, the $P_{10}VP$ medium was used as the basal medium supplemented with hymexazol at 25 and/or 50 μg/ml; the same medium without hymexazol was used as the control. Certain selected isolates were similarly tested using cornmeal agar as the basal medium. Test fungi were grown on cornmeal agar, removed as 4-mm-diameter agar disks and placed on test media in 90-mm-diameter plastic petri plates, usually with three different fungi

TABLE 2. Selective toxicity of hymexazol, incorporated at 50 μg/ml in the pimaricin-vancomycin-PCNB ($P_{10}VP$) medium, on in vitro growth of certain species and isolates of *Phytophthora*, *Pythium*, and *Mortierella*.

Test fungus	No. of tests	Avg. linear growth (with range in parentheses), expressed as a percentage of the growth of controls[a]	
Phytophthora cactorum (Leb. & Cohn) Schroet. (isolates 1 & 2)	5	53	(26.4-72.5)
P. cinnamomi Rands (isolates 1 & 2)	7	88.2	(75-100)
P. citrophthora (R. E. Sm. & E. H. Sm.) Leonian (isolates 1 & 2)	3	90.8	(72.3-100)
P. palmivora (Butl.) Butl., typical strain, morphological form 1 (isolates 1, 2, and 3)[b]	6	50.3	(33.3-73.7)
'*P. palmivora*', atypical black pepper strain (isolates 1 & 2)	3	86.5	(79-90.7)
P. parasitica Dast. (isolates 1 & 2)	3	75.5	(66.7-87.2)
Pythium aphanidermatum (Edson) Fitzp.	1	0	
P. irregulare Buis.	2	10.2	(8.7-11.7)
P. mamillatum Meurs	1	34.6	
P. oligandrum Drechs.	1	0	
P. periplocum Drechs.	1	15.4	
P. sylvaticum Campbell & Hendrix (isolates 1 & 2)	2	14.8	(7.6-22.1)
P. ultimum Trow	3	0	
P. sp. no. 1 (Py-3)	1	9.3	
P. sp. no. 2 (Py-7)	2	95	(94.2-95.8)
Mortierella alpina Peyronel	3	6.6	(0-12.7)
M. hyalina (Harz) W. Gams	2	0	
M. isabellina Oud. & Koning	2	...[c]	
M. ramanniana var. *angulispora* (Naum.) Linnemann	1	...[c]	
M. ramanniana var. *ramanniana* (Moell.) Linnemann	2	...[c]	
M. spinosa Linnemann	3	5.9	(0-17.8)
M. sp. no. 1 (M-4)	2	1	(0-2)
M. sp. no. 2 (M-6)	2	4.8	(0-9.6)
M. sp. no. 3 (M-B551)	3	10.3	(0-20.4)
M. sp. no. 4 (M-2C1)	2	0	
M. sp. no. 5 (M-14-4)	2	8.2	(7.6-8.8)

[a]Control medium was the $P_{10}VP$ medium without hymexazol. Linear growth was measured each day from the edge of agar disk to margin of colony as shown in Fig. 2. Each colony was measured three times in three different directions. Each figure in the table is an average of three replicate plates representing nine measurements at 2 or 3 days, except when there were more than one test per species, average of several tests is presented with range given in paranthesis.

[b]Isolates from cacao, papaya, and rubber were used; of the three, the cacao isolates was least sensitive to hymexazol.

[c]Growth of these species of *Mortierella* was completely inhibited on $P_{10}VP$ medium and therefore also on the same medium containing hymexazol.

sharing the same plate as shown in Fig. 2. Three replicate plates were used per isolate in each test. Plates were incubated at 25 C in the dark and linear growth (from edge of disk to margin of colony) was measured in three directions and recorded daily for 2 or 3 days. The density of colony growth also was noted. For most species, the test was repeated two to seven times.

RESULTS AND DISCUSSION

Recovery of Phytophthora from soil or roots and control of Mortierella and Pythium spp. on isolation medium containing hymexazol.—The $P_{10}VP$ medium containing hymexazol at 50 μg/ml (PVPH) effectively controlled the development of colonies of not only *Pythium*, but also *Mortierella* spp., in soil dilution plates with all five soils that were tested (Table 1, Fig. 1). Except for bacteria, which developed in relatively low members in both control and hymexazol media, the PVPH medium yielded almost pure stands of *P. cinnamomi* colonies whereas the same medium without hymexazol contained *Pythium* colonies and hundreds of *Mortierella* colonies per plate (Table 1, Fig. 1). Although *P. cinnamomi* appeared in slightly higher numbers on PVPH medium (Table 1), the difference in *Phytophthora* recovery in the two media were not significantly different ($P = 0.05$) in any of the five soils that were tested. However, *P. cinnamomi* colonies were more easily discerned and more accurately enumerated on the medium with hymexazol than without hymexazol (Fig. 1).

The direct inoculation, or soil-sprinkling, method was tested with six naturally infested avocado or citrus soils, all containing relatively low *Phytophthora* infestation (about 2 to 50 propagules/g soil). Again, the PVPH medium generally produced much cleaner plates than the $P_{10}VP$ medium without hymexazol. *Phytophthora cinnamomi* or *P. parasitica* colonies on PVPH medium, even when only one or two colonies per plate were present, stood out clearly among numerous soil particles against a background free of interfering *Pythium* and *Mortierella* colonies. The only exceptions were two naturally infested avocado soils where a *Pythium* sp. (isolate Py-7 in Table 2) developed in the plates and its colonies grew almost as much on PVPH medium as on control medium, attaining a colony diameter greater than 25 mm in 2 days. Results of the tests with root plating generally were similar to those with soil plating for most samples, including the two samples containing the insensitive *Pythium* sp. which also yielded *Pythium* growth on PVPH medium.

Selective toxicity of hymexazol to Phytophthora, Pythium, and Mortierella spp. in vitro.—A total of 34 species or isolated of *Phytophthora*, *Pythium*, and *Mortierella* were selected (Table 2) for in vitro evaluation of selective toxicity of hymexazol at 25 and/or 50 μg/ml. Results of inhibition at 50 μg/ml are summarized in Table 2. As reported by Masago et al. (5), the growth of most of the nine *Pythium* spp. tested was completely or greatly inhibited by the chemical at 50 μg/ml in the $P_{10}VP$ medium (Table 2). *Pythium* sp. no. 2 (isolate Py-7) was the only exception; it was almost totally insensitive to hymexazol at both 25 and 50 μg/ml when tested in either

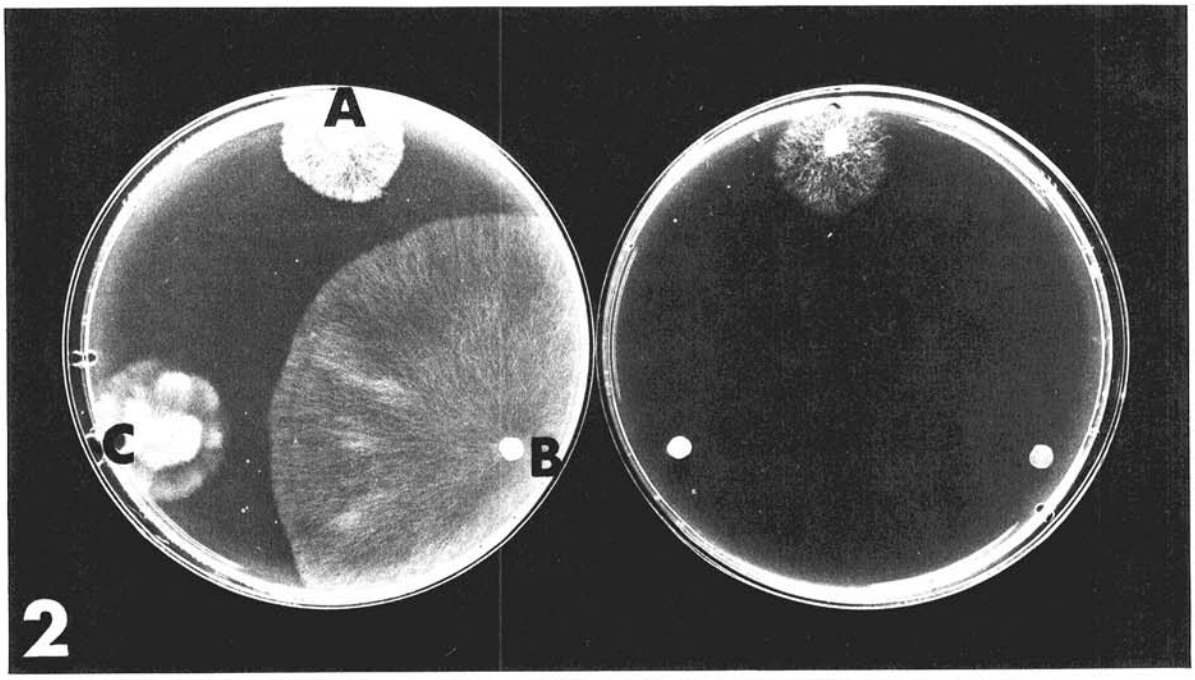

Fig. 2-(A to C). Selective toxicity in vitro of hymexazol to **A)** *Phytophthora cinnamomi*, **B)** *Pythium ultimum*, and **C)** a *Mortierella* sp. isolate M-4. **Left**, the pimaricin-vancomycin-PCNV ($P_{10}VP$) medium; **right**, the same medium containing 50 μg/ml hymexazol showing a slight reduction in linear growth and colony density of *P. cinnamomi* and complete inhibition of *Pythium* and *Mortierella* growth by hymexazol. Photographed at 2 days.

cornmeal agar or P_{10}VP medium. For example, its linear growth at 48 hr on P_{10}VP medium containing 0, 25, and 50 μg/ml hymexazol was 14.3, 14.7, and 13.7 mm, respectively; the growth density also was similar in the three media. Hymexazol at 25 μg/ml was not as inhibitory as at 50 μg/ml to some *Pythium* species. For example, linear growth of *Pythium mamillatum* at 48 hr on cornmeal agar containing 0, 25, and 50 μg/ml hymexazol was 28, 15, and 10 mm, respectively. With some other *Pythium* species, however, hymexazol at 25 μg/ml was almost as inhibitory as 50 μg/ml. For example, linear growth of *Pythium periplocum* at 48 hr on cornmeal agar containing 0, 25, and 50 μg/ml hymexazol was 43.2, 5.8, and 4 mm, respectively; and on P_{10}VP medium containing 0,25, and 50 μg/ml hymexazol it was 35.7, 6.4, and 5.5 mm, respectively. In general, colony growth of most *Pythium* spp. on media containing either concentration of hymexazol ranged from very sparse to almost nondiscernible.

When 11 *Mortierella* spp. or isolates were tested, only *M. isabellina*, *M. ramanniana* var. *angulispora*, and *M. ramanniana* var. *ramanniana* were completely inhibited on P_{10}VP medium, and therefore also inhibited on the same medium containing hymexazol. The other eight isolates grew substantially on P_{10}VP medium containing no hymexazol, but were greatly inhibited on P_{10}VP medium containing hymexazol at 50 μg/ml (Table 2); some isolates were inhibited even at 25 μg/ml. Hymexazol alone was not as inhibitory to *Mortierella* as when it was combined with other antimicrobial agents present in the P_{10}VP medium. For example, linear growth of *M. alpina* at 48 hr on cornmeal agar containing 0, 25, and 50 μg/ml hymexazol was 8.3, 3, and 1.3 mm, respectively; and on P_{10}VP medium containing 0, 25, and 50 μg/ml hymexazol it was 4.4, 0, and 0 mm, respectively. For *M. hyalina*, at the same respective concentrations, the growth was 10.4, 5.4, and 3.8 mm on cornmeal agar, and 3.2, 0, and 0 mm on the P_{10}VP medium. Thus, synergism between hymexazol and other toxicants was evident in these tests.

With few exceptions, growth of *Phytophthora* spp. was not greatly affected by hymexazol at 50 μg/ml (Table 2) and was even less inhibited at 25 μg/ml. All test isolates of *P. cinnamomi*, *P. citrophthora*, and *P. parasitica* grew well on P_{10}VP medium containing 50 μg/ml hymexazol with only slight or no reduction in growth rate and a slight reduction in colony density (*P. cinnamomi* in Fig. 2). Growth of *P. cactorum*, however, was greatly inhibited on the P_{10}VP medium containing hymexazol at 50 μg/ml, it being only 26.4-51.7% of that of the P_{10}VP control for one isolate and 72.5% of the control for the second isolate. Likewise, all three isolates (from cacao, papaya, and rubber) of the typical strain [morphological form 1 (15)] of *P. palmivora* tested were greatly inhibited with reductions that averaged about 50% (Table 2). Hymexazol at 25 μg/ml was almost equally inhibitory to *P. palmivora*; for example, linear growth of the rubber isolate at 48 hr on cornmeal agar containing 0, 25, and 50 μg/ml hymexazol was 12.4, 6.2, and 5.9 mm, respectively. In addition, colonies of both *P. cactorum* and *P. palmivora* on the hymexazol medium were extremely sparse. On the other hand, the so-called atypical black pepper strain of '*P. palmivora*' (15), which is likely a different species and has been named as *P. colocasiae* Raciborski by some workers (see citations in 15), responded quite differently to hymexazol from the typical *P. palmivora* isolates. Neither isolate of the black pepper strain tested was appreciably inhibited by hymexazol (Table 2).

The pimaricin-vancomycin-PCNB medium containing hymexazol at 50 μg/ml (PVPH medium) will likely perform satisfactorily for direct recovery of many *Phytophthora* spp. from soil and plant tissues. Based on the in vitro growth tests, some isolates of *P. cactorum* and *P. palmivora* are more sensitive to hymexazol and, therefore, may be more difficult to isolate than other isolates of the same species. Although the sparse colony growth of these and certain other *Phytophthora* isolates on the PVPH medium might hinder detection, successful control of *Pythium* and *Mortierella* development should more than compensate for this slight growth reduction. Nevertheless, for recovery of certain sensitive *Phytophthora* spp. from soil, P_{10}VP medium containing 25 μg/ml or less hymexazol might prove to be a more suitable isolation medium. In spite of the existence of the many available selective media for isolation of pythiaceous fungi, recovery of certain slow-growing *Phytophthora* spp., such as *P. fragariae*, from roots and soil remains difficult mainly because of abundant presence of *Pythium* and/or *Mortierella* spp. in infected tissues and infested soils. Incorporation of appropriate concentration of hymexazol in a proper selective isolation medium could greatly improve their chance of recovery. It is unfortunate that not all *Pythium* spp. are effectively inhibited by hymexazol and further testing of more soils and/or *Pythium* isolates might reveal additional insensitive species. Several derivatives of isoxazole are known to possess similar differential effects on pythiaceous fungi (5). One or more of these derivatives might selectively inhibit those *Pythium* spp. resistant to hymexazol. Their possible use as an additional selective agent in the PVPH medium will be investigated.

Mortierella, in the family Mortierellaceae of the order Mucorales, is a heterogeneous genus with most members having hyaline somatic and reproductive structures and a petaloid, rosette, or stellate colony appearance greatly resembling members of the Pythiaceae. However, some members in Section Isabellina (16), such as *M. isabellina* and *M. ramanniana*, have gray, pink, red, violet or other pigments and are totally unlike pythiaceous fungi in colony morphology. The latter group also has microscopic morphological features, such as columella, resembling species of *Mucor*; in fact, some members in the past have been placed in the genus *Mucor* (see 16). It is of interest that, toxicologically, *Mortierella* is the only genus in the entire order Mucorales which contains species insensitive to pimaricin and other polyene antibiotics (13). The differential sensitivity to pimaricin of *M. isabellina*, *M. ramanniana* and some other species from the rest of *Mortierella* spp. adds further support, chemotaxonomically, to the belief that not all species in this large and heterogeneous group belong in the same genus.

The *Pythium* isolate Py-7, which is insensitive to hymexazol at 50 μg/ml, has been identified recently by D. J. Stamps and O. Vaartaja as *Pythium vexans* d By.

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