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Ecologic and Taxonomic Studies on Pythium as Pathogenic Soil Fungi

VI The Isolation Methods of Pythium

By

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INTRODUCTION

Since so many organisms are in symbiotic or antagonistic relations in the soil, isolating soil-infecting fungi from bacteria, and other fungi is not easy.

A direct isolation from the soil and indirectly from diseased tissues could be considered as the methods of isolating soil-infecting fungi. But in both methods selective isolation of the fungus from associated bacteria and other fungi is necessary. It is ideal to use a suitable medium and method for selective isolation with due consideration on the species of the soil-infecting fungi.

From the above-mentioned points of view, TAKAHASHI (24) has pointed out that the following methods are important in isolating *Pythium* species from associated bacteria: (a) The use of culture media containing antibiotics; (b) changing cultural conditions; (c) sterilization or disinfection of diseased tissues; and (d) use of culture media which are unsuitable for bacterial growth. He also listed such selective isolations of *Pythium* species from associated fungi by the differences in physiological characteristics; by ecology in soil and by the differences in growth rate on culture media containing antibiotics.

Both direct and indirect methods are commonly used in the isolation of soil-infecting fungi from associated bacteria and fungi by using culture media containing antibiotics. The soil-infecting fungi were protected from associated bacteria by the use of rose bengal and 30-100 ppm streptomycin (13, 14, 17). *Pythium* species were isolated without bacterial contamination by adding 5 ppm endomycin and 50 ppm streptomycin to the basal synthetic medium (21). *Rhizoctonia solani* was isolated using a culture medium with a piece of fagpyron as a nutrient source and containing 50 ppm each of aureomycin, neomycin and streptomycin (20). *Verticillium albo-atrum* REINKE & BERTH. isolated by using the agar medium containing streptomycin and 5,500 ppm alcohol (18). Plenty of papers have been published on the effectiveness of pimaricin to selective isolation of soil-infecting fungi from associated bacteria and fungi. Pimaricin was first used for the isolation of *Phytophthora infestans* and the growth of fungal contaminants were supressed by adding 100 ppm Na-salt of pimaricin (5). *Pythium* species were isolated by the addition of pimaricin to culture media (23). Nystatin and pimaricin were effective for isolation of *Pythium* species (22), and they were also effective for isolation of *Phytophthora* and *Pythium* species from associated bacteria and fungi (6). The isolation of *Pythium* species and *Phytophthora* was conducted successfully by using the culture medium containing 100 ppm pimaricin (9). Such antibiotics as penicillin G (2), aureomycin (4,8,12), and novovicin (11) are also used for the same purpose.

In addition to antibiotics, it has been reported that fungicides would inhibit the growth of such fast growing fungi as *Rhizopus*, *Trichoderma*, *Penicillium* and *Verticillium*. The culture medium containing PCNB is used to suppress the faster growth of *Rhizopus stolanifer*

(Ehr. ex Fr.) LINDEN than any other fungi in the same test tube (1). Thirty ppm of rose bengal is effective for suppressing the growth of bacteria and actinomycetes; 4 ppm PCNB is effective for *Rhizopus*, and 20 ppm duramycin is effective for *Trichoderma* (25). *Fusarium sp.* was selectively isolated from *Rhizopus* by adding 100-500 ppm of PCNB to the soil (15). Crystal violet (13), or oxgall (16) is effective in reducing the rate of growth in fast growing fungi such as *Rhizopus*, *Trichoderma*, etc.

In the present paper, the isolation of *Pythium* species from soil-borne microorganisms such as bacteria and fungi using the culture media containing antibiotics and soil fungicides was studied.

MATERIALS AND METHODS

Ten antibiotic preparations with 20 and 50 ppm were made from streptomycin "Sankyo", penicillin G, terramycin hydrochloride, kanamycin, actidione, streptomycin + penicillin, streptomycin+terramycin, streptomycin+kanamycin, penicillin+terramycin and actidione + streptomycin.

Individual preparations were mixed with melted corn-meal agar (CMA) after cooling down to about 60°C. and poured in sterile Petri dishes. After the agar has solidified the dishes were streaked with *Bacillus subtilis* which is Gram positive and other dishes with *Bacillus aroides*, a Gram negative. Control dishes were made with the medium only but also streaked with the same test organisms. The dishes were incubated at 28°C. After the appearance of growth in the control dishes evaluation of the given antibiotics was determined. It was indicated with a sign (+) if the antibiotic failed to inhibit completely the growth of the test organisms and (−) sign if the bacterial growth was completely inhibited.

The concentrations of the antibiotics that inhibited the test organisms in Method I were used in determining the effect on *Pythium* and other 3 soil-borne pathogens, namely, *Pythium aphanidermatum*, *P. arrhenomanes*, *P. debaryanum*, *P. mamillatum*, *P. spinosum*, *P. ultimum*, *P. vexans*, *P. zingiberum*, *Rhizoctonia solani*, *Fusarium oxysporum* f. *lycopersici* and *Sclerotium rolfsii*.

Bit of agar approximately 5 mm square, containing young culture of the test organisms was placed at the center of each dish with the desired concentration of the antibiotic. The dishes were incubated at 28°C. for 24-48 hours. The effect of antibiotic on fungal growth was determined by measuring the diameter of the ramifying hyphae. The same procedures were followed in the control.

The effects of three chemically different soil fungicides, namely, actidione, Ethyl Mercuric Phosphate (EMP) and Pentachloronitrobenzen (PCNB) at varying concentrations were also determined. The actidione was prepared at 0.01, 0.1, 1.0, 10.0 and 50 ppm; the EMP with 0.001, 0.01, 0.1, 1.0 and 10 ppm and PCNB with 50, 200, 400, 500 and 1000 ppm. Another preparations were made with the same fungicides and concentrations but with the addition of streptomycin at 50 ppm level.

The effects of peptone-dextrose agar plus 30 ppm rose bengal and 50 ppm streptomycin (PDA-S50-RB30) which was used by JOHNSON (12) in isolating soil fungi employing the dilution plate method to the above-mentioned fungi were also determined by measuring the diameter of the ramifying hyphae. However, 500 ppm PCNB was added to PDA-S50-RB30 and the same procedures were followed.

The pathogenic fungi used in Method II were separately cultured on wheat bran medium (wheat bran, 20 g., distilled water 10 ml) in flasks for 2-4 days at 28°C. After the incubation, 50 g. of sterile garden soil and 10 ml sterile distilled water were added to the culture flasks and then incubated further for 2 more days. The cultures were jarred to shake the soil and break the lumps thereby cutting the mycelium into fragments and finally sieved in 2 mm screen mesh. After sieving Pythium were mixed together thoroughly. About 1 gram

of the mixed cultures was used to inoculate the center of each dish containing CMA with 50 ppm streptomycin. Microscopic examination was done 2 days after incubation to identify the species that survived the treatment.

Antagonistic phenomenon between *Pythium* and *Rhizoctonia solani*.

Several sterile Petri dishes were plated with 10 ml of potato dextrose agar. On one side of the dish small bit of agar with *Rhizoctonia solani* was introduced. The dish was incubated for 16 hours and then each of *P. aphanidermatum*, *P. arrhenomanes*, *P. ultimum*, and *P. zingiberum* were introduced on the opposite side approximately 6 cm from *Rhizoctonia*. Another *Pythium*, *P. debaryanum*, *P. mamillatum*, *P. spinosum* and *P. vexans* were seeded at the same time with *Rhizoctonia*.

An effects of culture filtrates of *Pythium ultimum* and *Rhizoctonia solani* to the same organism.

Four different media were used in this method.

1) Corn-meal decoction. The 20 g corn meal was boiled in water bath for an hour in 500 ml of distilled water and filtered through cheesecloth. Another 500 ml of distilled water with 20 g of dextrose was added to the corn meal filtrate and the volume was adjusted to 1 liter, dispensed in flasks and sterilized at 15 lbs for 15 minutes.

2) Potato decoction. The potato with 500 ml distilled water was boiled in a steamer for an hour or 40 minutes in the autoclave and filtered through cheesecloth. To the filtrate was added 500 ml of distilled water with 20 g of dextrose. The solution was finally adjusted to 1 liter, dispensed in flaskes and sterilized at 15 lbs. for 15 minutes.

3) *Richard's* medium. All the chemicals were dissolved in 500 ml of distilled water and then adjusted to 1 liter, dispensed in flasks and sterilized at 15 lbs for 15 minutes.

4) *Misato's* medium. The yeast extract was dissolved in hot water with intermittent stirring, starch added and the volume was restored to 1 liter, dispensed in flasks and sterilized at 15 lbs for 15 minutes.

Pythium ultimum and *Rhizoctonia solani* were cultured in these 4 different media for 30 days at 28°C. The media were revitalized by adding fresh medium to each culture after 30 days of incubation and then incubated further for 10 more days. The effect of culture filtrates on the growth of both fungi was determined by comparing the dry weight of the mycelia with that of the control.

EXPERIMENTAL RESULTS

I. Bacterial growth on corn-meal agar containing antibiotic substances.

Bacterial growth is inhibited on CMA containing 20 and 50 ppm of all antibiotic preparations used except with 20 ppm terramycin and kanamycin and 20 and 50 ppm actidione (Table 1).

It proves that the bacterial growth can be inhibited by using CMA containing 50 ppm or thereabout concentrations of streptomycin and penicillin.

II. Selective growth of *Pythium*, *Rhizoctonia solani*, *Fusarium oxysporum* f. *lycopersici* and *Sclerotium rolfsii* on media which inhibit the bacterial growth.

Pythium ultimum can be isolated from bacteria as shown in Table 2 by using CMA containing 50 ppm of the antibiotics. Corn-meal agar containing antibotics such as 20 and 50 ppm streptomycin + terramycin and streptomycin + kanamycin and 50 ppm penicillin + terramycin, however, inhibits the growth of *Pythium ultimum*. On the other hand, on CMA containing 20 and 50 ppm streptomycin and penicillin, *P. ultimum* grows a little better than the control (Table 2).

Using CMA containing 50 ppm streptomycin and penicillin which inhibited bacterial growth without inhibiting growth of *Pythium ultimum*, growth of the other *Pythium* was also studied. Consequently, though on CMA containing 50 ppm streptomycin, *Pythium*

Table 1. Bacterial growth on CMA containing different concentrations of antibiotic(s).

Antibiotic	Concentration (ppm)	TEST ORGANISMS	
		Bacillus subtilis	*Bacillus aroideae*
Streptomycin	20	−	−
	50	−	−
Penicillin	20	−	−
	50	−	−
Terramycin	20	+	+
	50	−	−
Kanamycin	20	+	+
	50	−	−
Streptomycin+Penicillin	20+20	−	−
	50+50	−	−
Streptomycin+Terramycin	20+20	−	−
	50+50	−	±
Streptomycin+Kanamycin	20+20	−	−
	50+50	−	−
Penicillin+Terramycin	20+20	−	−
	50+50	−	−
Streptomycin+Actidione	20+20	+	±
	50+50	−	−
Actidione	20	+	+
	50	+	+

NOTE: −=no growth +=with growth ±=growth partially suppressed

Table 2. Selective growth of 4 soil-borne fungi in CMA containing different concentrations of antibiotic(s).

Antibiotic	Concentration (ppm)	P. *u.*	*R. s.*	*S. r.*	*F. oxys. f. lyco.*
		*	**	**	***
Streptomycin	20	4.90	5.70	4.65	3.70
	50	4.70	5.80	4.70	3.40
Penicillin	20	5.20	5.35	4.90	3.80
	50	5.00	4.50	4.95	4.00
Terramycin	20	3.70	5.10	4.80	3.85
	50	2.85	4.60	4.55	3.20
Kanamycin	20	3.00	5.90	4.65	3.45
	50	3.40	4.60	4.75	3.55
Streptomycin+Penicillin	20+20	4.30	6.25	4.60	3.65
	50+50	4.50	4.05	4.45	3.90
Streptomycin+Terramycin	20+20	2.45	5.10	5.25	4.00
	50+50	1.75	4.55	4.90	3.65
Streptomycin+Kanamycin	20+20	1.95	5.30	4.60	3.60
	50+50	1.75	5.05	4.75	3.60
Streptomycin+Actidione	50+50	0	0	0	1.60
Penicillin+Terramycin	20+20	4.90	5.60	5.10	4.30
Control	50+50	2.15	5.10	4.90	3.90
	— —	4.50	5.90	5.45	3.80

NOTE: *P. u.*=*Pythium ultimum* *R. s.*=*Rhizoctonia solani* *S. r.*=*Sclerotium rolfsii*
F. oxys. f. *lyco.*=*Fusarium oxysporum* f. *lycopersici*
*=Mean diameter of hyphae after 24 hours at 28°C.
**=Mean diameter of hyphae after 48 hours at 28°C.
***=Mean diameter of hyphae after 72 hours at 28°C.

arrhenomanes and *P. vexans* did not grow well, all other *Pythium* grow better than the control. The CMA containing 20 or 50 ppm streptomycin or penicillin seems to be suitable additive for the media to inhibit the bacterial growth without inhibiting growth of *Pythium*. With regards to *P. arrhenomanes* and *P. vexans* although they are inhibited to a lesser extent by the streptomycin they are seemingly favored by CMA containing penicillin.

Table 3. Growth of *Pythium* on CMA containing 50 ppm of antibiotic. (Measurements in cm).

Pythium	Streptomycin		Penicillin		Control	
	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
Pythium aphanidermatum	7.85	+++	+	+++	7.75	+++
P. arrhenomanes	3.90	5.90	6.85	++	7.20	+++
P. debaryanum	4.35	+	4.85	++	4.40	++
P. mamillatum	4.25	+	5.05	++	4.60	+
P. spinosum	5.50	+	5.45	+	5.90	++
P. ultimum	4.70	+	4.85	+	4.65	++
P. vexans	4.90	+	5.80	++	6.10	++
P. zingiberum	6.25	+	6.15	+	5.60	+

NOTE: + =good growth
++ =better growth
+++=best growth

III. Selective growth of *Pythium* and other 3 soil-borne fungi on CMA containing different concentrations of several kinds of soil fungicides.

1. Growth of 4 soil-brone fungi on CMA containing actidione.

Table 4. Selective growth of 4 soil-borne fungi on CMA with different concentrations of soil fungicides and 50 ppm streptomycin. (Measurements in cm).

Fungicide and Concentration (ppm)	*P. ultimum*	*R. solani*	*F. oxysporum* f. *lycopersici*	*S. rolfsii*
PCNB-1000	3.80 (2.50)	− (−)	± (±)	− (−)
500	++	2.20	±	−
400	++	5.80	2.70	3.50
200	++	6.20	2.50	3.80
Actidione				
100	−	−	−	−
50	− (−)	− (−)	1.70 (1.40)	− (−)
10	0.8 (1.2)	1.0 (1.0)	2.20 (2.20)	− (−)
1.0	++	+	+	+
EMP 0.1	5.60 (4.80)	3.90 (2.20)	2.5 (1.90)	± (±)
0.01	++ (4.30)	3.70 (3.60)	2.60 (2.10)	2.80 ±
0.001	++	3.90	2.90	3.50

NOTE: +=no aerial growth; ++=with aerial growth; −= no growth
±=growth is partially restricted
( )=figures in parenthesis are data obtained with streptomycin.

As shown in Table 4, only *Fusarium oxysporum* f. *lycopersici* grows specifically on CMA containing 50 ppm of actidione. Another fungi including *Pythium ultimum* are inhibited in

the medium. The same results are obtained by using the same fungicide and concentrations but with the addition of streptomycin at 50 ppm. The results suggest that *Fusarium oxysporum* f. *lycopersici* can be isolated by using CMA containing actidione at 50 ppm and streptomycin at 50 ppm (CMA-S50-A50). The results also show CMA-S50-A50 is not suitable for isolation of *P. ultimum*.

2. Growth of 4 soil-borne fungi on CMA containing EMP.

As shown in Table 4 no fungi grow specifically on CMA containing EMP at varying concentrations. The same results are obtained by using the medium containing EMP mixed with streptomycin at 50 ppm. Again it shows that the medium using the EMP is not suitable for isolation of *P. ultimum* and other *Pythium*.

3. Growth of 4 soil-borne fungi on CMA containing PCNB.

Pythium ultimum grows specifically on CMA containing PCNB at 1000 ppm though its growth is somewhat inhibited by PCNB (Table 4). The same result is obtained when streptomycin at 50 ppm was added to CMA containing PCNB at 1000 ppm (CMA-S50-P1000). The other fungi, *Rhizoctonia solani*, *Fusarium oxysporum* f. *lycopersici* and *Sclerotium rolfsii* are not able to grow on CMA-S50-P1000.

Table 5. Growth of *Pythium* on CMA containing 50 ppm streptmoycin and 1000 ppm PCNB.

Pythium	INCUBATION HOUR (DIAMETER IN CM.)									
	24		48		72		96		120	
	P1000	S50	P1000	S50	P1000	S50	P1000	S50	P1000	S50
P. aphanidermatum	±	1.10	1.20	1.50	2.05	2.50	2.45	3.15	2.75	3.80
P. arrhenomanes	±	−	−	−	−	−	±	±	±	1.10
P. debaryanum	1.10	1.20	2.35	2.00	3.90	2.95	4.25	3.60	5.00	4.60
P. mamillatum	−	−	1.55	−	2.40	−	3.10	±	3.65	1.25
P. spinosum	±	±	1.95	1.65	3.05	3.10	3.70	3.65	4.35	4.05
P. ultimum	1.80	±	3.80	2.10	++	4.90	++	++	++	++
P. vexans	±	1.00	1.60	2.20	2.75	3.40	3.70	3.60	4.35	4.60
P. zingiberum	1.90	1.70	3.65	3.70	+	+	+	+	+	+

NOTE: + =no aerial hyphae
++=with aerial hyphae
± =growth partially restricted
− =no growth

As shown in Table 5 most of *Pythium* except *P. arrhenomanes* and *P. mamillatum* are able to grow on CMA with 1000 ppm PCNB.

The results may indicate that *Pythium* can be isolated from bacteria and other soil-borne fungi by using CMA-S50-P1000.

IV. Growth of *Pythium* and other soil-borne fungi on peptone dextrose agar containing 30 ppm rose bengal, rose bengal + 50 ppm streptomycin and rose bengal + streptomycin + 500 ppm PCNB.

As what has been previously observed *Pythium* are somewhat inhibited by PCNB so another medium plus several chemicals combinations was made to determine the effects on the growth of *Pythium* (Table 6).

The growth of *Pythium* as well as *Rhizoctonia solani*, *Fusarium oxysporum* f. *lycopersici* and *Sclerotium rolfsii* is better than the control on peptone dextrose agar containing rose bengal and rose bengal + streptomycin. This shows, therefore, that both medium are not suitable for selective isolation of *Pythium*. On the other hand, *Pythium* except for *P. arrhenomanes* and *P. debaryanum* grow specifically on peptone dextrose agar containing

Table 6. Growth of *Pythium* and 3 other soil-borne fungi on peptone-dextrose agar containing 30 ppm rose bengal (a), rose bengal + 50 ppm streptomycin (b), and rose bengal + streptomycin + 500 ppm PCNB (c).

Test Fungi and treatment		INCUBATION HOUR				
		24	48	72	96	120
Pythium aphanidermatum						
	(a)	2.60	5.05	7.30	+++	+++
	(b)	2.35	4.55	6.55	+++	+++
	(c)	±	1.60	2.30	3.25	3.90
P. arrhenomanes						
	(a)	3.25	6.00	7.80	+	+
	(b)	3.10	4.95	6.55	8.10	+
	(c)	−	±	1.10	1.25	1.60
P. debaryanum						
	(a)	1.65	3.30	4.60	7.35	+
	(b)	1.55	2.05	3.45	4.35	6.20
	(c)	−	±	±	1.25	1.75
P. mamillatum						
	(a)	2.55	5.15	6.75	++	++
	(b)	2.55	4.25	5.70	++	++
	(c)	−	1.25	1.65	2.80	3.20
P. spinosum						
	(a)	2.45	5.60	7.20	+	+
	(b)	2.25	4.10	6.05	+	+
	(c)	−	1.10	1.80	2.50	2.90
P. ultimum						
	(a)	1.90	3.15	4.10	5.80	++
	(b)	1.85	3.00	4.05	6.20	++
	(c)	±	1.40	1.65	2.10	2.60
P. vexans						
	(a)	1.25	2.30	3.60	6.35	+
	(b)	1.75	3.55	4.50	5.90	+
	(c)	−	1.00	1.20	1.90	2.20
P. zingiberum						
	(a)	1.90	3.20	4.45	7.30	+
	(b)	1.85	3.25	4.45	7.00	+
	(c)	1.10	2.15	2.90	4.00	5.10
Rhizoctonia solani						
	(a)	1.70	3.25	4.35	7.40	+
	(b)	1.85	2.85	4.75	7.95	+
	(c)	−	±	±	1.35	1.50
Fusarium oxysporum f. *lycopersici*	(a)	±	1.40	1.65	2.25	0
	(b)	±	1.25	1.60	2.30	0
	(c)	−	±	1.15	1.55	1.70
Sclerotium rolfsii						
	(a)	1.35	3.10	4.25	6.90	0
	(b)	1.60	3.60	5.00	7.60	0
	(c)	−	±	±	±	±

NOTE: − =no growth
+ =no aerial growth
++=with aerial growth
± =growth partially restricted
0 =no data obtained

rose bengal, streptomycin and 500 ppm PCNB (PDA-S50-RB30-P500). This medium may be suitable for isolating *Pythium* from bacteria and other soil-borne fungi the same as CMA-S50-P1000.

V. Isolation method of a few soil-borne fungi from the soil.

In the first part of this investigation CMA containing different concentrations of antibiotic substances and soil fungicides growth of *Pythium* and other three soil-borne fungi on some media which seems to be suitable for isolation work at the exclusion of bacteria and other soil-borne fungi were found.

In this experiment, using media suitable for isolation of a few fungi in previous investigation is determined whether the fungi can be isolated from the sterilized and non-sterilized soils infested with 4 pre-cultured fungi.

1. Growth of *Pythium* and 4 other soil-borne fungi cultured in sterilized soil on CMA containing the same concentrations of soil fungicides and 50 ppm streptomycin are investigated individually. Remarkably the same results are obtained as shown in Tables 4 and 5. The organisms either precultured on CMA or in soil, the growth is the same. Then the same number and amount of *Pythium* species and three other fungi cultured in soil are mixed and selective isolation of each fungi are conducted using CMA-S50-P1000 and PDA-S50-P500 which were previously found to be specific on *Pythium*, CMA-S50-A50 and CMA-S50 specific to *Fusarium oxysporum* f. *lycopersici*.

All the *Pythium* used with the exception of *Pythium arrhenomanes* and *P. mamillatum* grow on CMA-S50-P1000 and except *P. arrhenomanes* and *P. debaryanum* grow on PDA-S50-RB30-P500; only *Fusarium oxysporum* f. *lycopersici* grows on CMA-S50-A50 while the other fungi used failed to grow in these media. *Pythium* which were found to have the faster rate of growth among the organisms used are expected to be isolated when CMA-S50 is used. However *Pythium* failed to grow while *Rhizoctonia solani* does.

2. Antagonistic phenomenon between *Pythium ultimum* and *Rhizoctonia solani*.

As was found in previous investigations *Rhizoctonia solani* inhibits the growth of *Pythium ultimum* on CMA-S50. In view of the above observations it is necessary to know whether antagonistic phenomenon between *Rhizoctonia* and *Pythium* exists.

Two days after incubation the hyphae of both organisms planted on the opposite sides mets at the center of the Petri dishes but further growth of *P. ultimum* is inhibited completely. Hyphae of *Rhizoctonia solani* spreads over the *P. ultimum*. The same results were also found with other *Pythium*.

3. Effects of culture filtrates on the growth of the organisms.

The culture filtrate of *Rhizoctonia solani* inhibits moderately the growth of *P. ultimum* compared with the control. The culture filtrates of *P. ultimum* and *Rhizoctonia solani* also slightly inhibit the fungi itself. It will require further examination to know whether the results are due to the toxin produced by both organisms. But since the growth of *P. ultimum* in culture filtrate of *Rhizoctonia solani* is considerably poor compared with both control and its culture filtrate, *P. ultimum* seems to be inhibited due to toxin which is produced by *Rhizoctonia solani*. Concerning toxin production by *Rhizoctonia solani*, NISHIMURA (19) and BATEMAN (3) have reported that pectin amelolytic enzyme causing the breaking of tissues and an enzymatic substances causing the necrosis of tissues were produced by the organism. But whether these substances are responsible to partial inhibition of growth of *Pythium* still necessitates further studies.

4. Selective isolation of the organisms.

The mixture of *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium oxysporum* f. *lycopersici* and *Sclerotium rolfsii* was cultured in non-sterilized soil which was regarded as a natural environment. The media used for selective isolation were CMA-S50; CMA-S50-P1000; PDA-S50-RB30-P500 and CMA-S50-A50. *Pythium ultimum* was isolated by using CMA-S50-P1000 and PDA-S50-RB30-P500 and *Fusarium oxysporum* f. *lycopersici* by CMA-S50-

A50. Furthermore it was found that *P. ultimum* can be isolated by using CMA-S50 in the absence of *R. solani* which antagonize it using the difference in speed of hyphal growth. If *R. solani* contaminate *P. ultimum*, CMA-S50-P1000 which inhibits *R. solani* specifically will be effective in isolating *P. ultimum*. *Rhizoctonia solani* can be isolated from fast growing fungi like *Rhizopus* with the use of CMA-S50-P500. Other *Pythium* such as *P. aphanidermatum*, *P. spinosum*, *P. vexans* and *P. zingiberum* can be isolated by using CMA-S50-P1000 and PDA-S50-RB30-P500. These media inhibit the growth of bacteria, actinomycetes and other soil-borne fungi. It must be noted, however, that *P. arrhenomanes*, *P. debaryanum* and *P. mamillatum* are inhibited by both media.

DISCUSSIONS

Culture medium for isolating soil-infecting fungi should generally have the following requirements: (a) must support good growth, (b) inhibits the multiplication of contaminants, especially bacteria (c) suppress the growth of contaminating fungi.

GOLDBERG (7), ECKERT, et al (6) listed the following criteria for antibiotics useful in selective media for the isolation and cultivation of microorganisms: (1) stability in the medium, (2) solubility in the medium, (3) a highly specific spectrum, and (4) freedom from toxicity for the organism to be cultivated.

Corn-meal agar containing 1000 ppm PCNB and 50 ppm streptomycin (CMA-S50-P1000) in this experiment answers all of the above mentioned criteria and is a suitable medium for isolating *Pythium* species from soil microorganisms.

The following antibiotics are commonly use for the isolation of soil-infecting fungi from bacteria: streptomycin, pimaricin, penicillin, aureomycin, novobiocin, endomycin, nystatin and another chemical but not antibiotic, rose bengal. With the exception of 20 ppm terramycin and 20 ppm kanamycin all antibiotics at 50 ppm used are good for the isolation of soil-infecting fungi. The growth of *Pythium* is suppressed, however, by 50 ppm streptomycin mixed with 50 ppm terramycin and the 20 ppm streptomycin mixed with 50 ppm kanamycin. The growth of the fungus to be isolated was suppressed before the growth of the contaminating bacteria was inhibited. Thus these combinations does not fit the criteria previously cited. It is, therefore, important to select the suitable antibiotics and its useful concentrations for the organism to be isolated. To a greater extent streptomycin at 50 ppm appears to be the ideal antibiotic except for *Pythium mamillatum* and *P. arrhenomanes* (10). Using 50 ppm of penicillin may be more suitable for these two *Pythium*.

The fungicide PCNB which is known to be effective to *Rhizoctonia* is also effective against the fast-growing fungi such as *Rhizopus*.

The result that only *Pythium* grows specifically on the culture medium containing 1000 ppm PCNB indicates that PCNB is a good chemical for the isolation of *Pythium* especially if the source is contaminated with *Rhizoctonia*. Since 1000 ppm PCNB inhibits not only *Rhizoctonia* but also some other fungi it is probable to isolate *Pythium* selectively by taking 50 ppm streptomycin, which inhibits bacteria and with 1000 ppm PCNB, which will check the fungal contaminants.

SUMMARY

Selective media for the isolation of *Pythium* such as *P. ultimum*, *P. aphanidermatum*, *P. spinosum*, *P. vexans*, and *P. zingiberum*, and other soil-borne pathogens like *Fusarium oxysporum* f. *lycopersici*, *Rhizoctonia solani* and *Sclerotium rolfsii* were found.

The media found ideal for the isolation of the specific organisms free from bacterial contamination as well as other fungi are the CMA-S50-A50, CMA-S50-P1000, PDA-S50-RB30-P300 and CMA-S50-P500.

Streptomycin and penicillin at 50 ppm have been found to reduce bacterial contamination without inhibiting growth of *Pythium*. Moreover, PCNB at a concentration of 1000 ppm in corn-meal agar eliminates other fungi without adversely affecting the development of *Pythium*. MARTIN's peptone-dextrose-rose bengal agar with 500 ppm PCNB has been found also to reduce greatly the number of contaminating fungi and actinomycetes.

Pythium aphanidermatum, *P. spinosum*, *P. ultimum*, *P. vexans*, and *P. zingiberum* were isolated from bacteria, actinomycetes and other soil-borne fungi by using corn-meal agar containing 50 ppm streptomycin and 1000 ppm PCNB. Peptone-dextrose agar containing 30 ppm rose bengal, 50 ppm streptomycin and 500 ppm PCNB are also effective media for the isolation of *Pythium* from soil-borne microorganisms.

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