ABUNDANCE OF *PYTHIUM* SPECIES IN NURSERY SOILS IN SOUTH AUSTRALIA

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Summary

The abundance of *Pythium* propagules and frequency of 11 *Pythium* species were estimated in 16 soil plots in South Australian forest nurseries, in one ornamental nursery, and in one pine forest. A soil-plating technique was utilized and a medium developed which was highly selective for these species and allowed their identification directly on the plates. The number of *Pythium* propagules in a unit volume of soil varied greatly, being lowest (24 per cubic centimetre) in the forest and highest (226 per cubic centimetre) in an alkaline loamy nursery soil. Loams had significantly more *Pythium* propagules than sandy soils. The immediately previous usage and soil amendments usually made no great difference to the total number of *Pythium* propagules. It is concluded that the abundance of pathogenic *Pythium* spp. in these nurseries is not the main factor governing the great variation found in the disease incidence of conifer seedlings. *P. irregulare* Buisman was frequent in all plots except the forest, and *P. mamillatum* Meurs in most sandy nurseries. The frequencies of *P. ultimum* Trow, *P. paroecandrum* Drechsler, *P. rostratum* Butler, *P. iwayamai* Ito, *P. oligandrum* Drechsler, *P. acanthicum* Drechsler, and *P. echinulatum* Matthews varied inconsistently between the plots. A new species, coded as *P. col*, was rare but occurred in eight plots. Another new species (*P. dis*) occurred only in one plot but frequently in this. This was a nursery freshly established on grassland pasture which differed from the others also in the frequencies of several other species.

I. INTRODUCTION

Until recently it has been difficult to reveal the true numbers of *Pythium* propagules in soil. The numerous other fungi present tend to inhibit *Pythium* and mask the population of this fungal group in dilution and soil platings. Screening tests with various antibiotics, commercial fungitoxicants, and other chemicals suggested that several of these might be useful in developing selective media for *Pythium* (Vaartaja 1960). Polyene antibiotics, such as pimaricin and endomycin, were found especially selective and have recently been utilized in isolation of *Pythium* (Singh and Mitchell 1961; Schmitthenner 1962; Kerr 1963). These and the earlier methods were reviewed by Schmitthenner (1962). The media would be most useful if they allowed identification of species directly on the isolation plates. This was partially achieved by Schmitthenner (1962).

The need to intensify the study of the occurrence of *Pythium* in South Australian nurseries became particularly urgent in 1961 when there were several serious epidemics in pine beds. *Pythium* species were commonly isolated from dying seedlings in all epidemics. Large numbers of seedlings of *Pinus pinaster* Ait. and *P. radiata* D. Don

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died in soils in which damping-off risk is usually thought to be small. Such epidemics occurred in poor, somewhat acidic, sandy soils, which included areas mulched with pine needle litter, other areas not previously used as a nursery, and areas that were freshly cleared from pine forests (plantations). On the other hand, the disease appeared to be reduced in plots into which large amounts of sawdust or pine bark had been mixed. The hypothesis was advanced that *Pythium* was abundant in all such soils and that the disease severity was determined by factors affecting either the activity of the inoculum or susceptibility of seedlings, or both.

This paper describes studies of the abundance of *Pythium* species in forest nurseries, utilizing a highly selective isolation medium.

II. Methods

(*a*) *Plots*

In each nursery or plot about 100 g of soil was taken at each of about 10 sites at equal distances along a line in the centre of an area with uniform contour and soil. This area was selected also as representative, if possible, of uniform and severe disease in the seedling stand. Samples were collected during late summer and included surface soil to a depth of 3–4 in. This is the depth where seedling lesions were common and at which Warcup (1952), in England, found *Pythium* species to be abundant. All samples from a plot were put together, mixed thoroughly, and stored in plastic bags at 0°C for periods varying from 2 to 10 weeks until studied. Because *Pythium* in soil plates grows mainly from resting structures (Warcup 1952), moderately long storage probably does not affect the results. Such was the case in the experiments of Schmitthenner (1962) even after the soil had been kept dry for 16 days.

It was realized that this composite sampling would give misleading results if the distribution of *Pythium* propagules were grossly uneven. The method was chosen because the distribution was likely to be relatively even, as judged from uniformity of the soil and from disease incidence in each area. Splash and run-off during irrigation and rain, common drifting of soil with wind, regular hoeing, and the yearly lifting of seedlings presumably combine to cancel out variation in population density of *Pythium* species. Warcup (1952) did not find marked variation between 0·25-g samples of soil taken 1 yd apart in English nurseries. This problem in general is further discussed by Johnson *et al.* (1959). Studying to what degree irregular distribution might occur is interesting but involves considerable work as each final sample studied cannot be more than about 0·1 cm^3 of soil and a nursery may have an area of several acres. On the other hand, it was important to obtain an indication of the presence of *Pythium* species in each of many plots and whether *Pythium* incidence would vary greatly between plots. The method was satisfactory for this purpose. The variety of plots sampled is briefly characterized in Table 1.

(*b*) *Plating*

The abundance of *Pythium* populations in the soil samples was estimated utilizing Warcup's (1950) "soil plate" method with 30 plates from the composite

sample of each plot. 0·07 cm³ of soil taken at random was spread in each plate and mixed with cooled but still molten agar of the following composition:

Water	1000 cm³	Agar	15 g
$NaNO_3$	2 g	Sucrose	1·5 g
KH_2PO_4	1 g	Rose bengal	50 mg
KCl	0·5 g	Mycostatin*	500 mg
$MgSO_4.x7H_2O$	0·5 g	Pentachloronitrobenzene	15 mg
$FeSO_4$	0·1 g	Streptomycin sulphate	50 mg
Yeast extract	0·5 g		

* Supplied by E. R. Squibb and Sons, Melbourne.

Various other recipes were tried but many allowed fairly rapid growth of other fungi, especially of *Mortierella* spp. Others made the *Pythium* colonies either difficult to see at all or too dense for identification directly on the isolation plate. It was observed that the high content of mycostatin stimulated production of oogonia in certain isolates of *Pythium*.

Most *Pythium* colonies spread rapidly and were easy to record on the third day after plating. Sample cultures showed that most of the slower colonies were species of bacteria, *Penicillium*, *Fusarium*, or *Mortierella*. Their further growth was suppressed by pouring a 0·03% aqueous solution of mycostatin on the plates on the fourth day, after subculturing a number of colonies for identification. The plates were kept in darkness to avoid decomposition of rose bengal and mycostatin. A few colonies of *Mortierella* developed even under these conditions, although slowly. Numerous sample colonies were subcultured and examined later to estimate the number of *Mortierella* colonies recorded amongst *Pythium* colonies. The necessary corrections were then made. Colonies of other fungi were not mistaken as possible *Pythium* species.

III. Results

(*a*) *Abundance of* Pythium *Propagules*

The average numbers of *Pythium* colonies obtained from different plots are shown in Table 1. *Pythium* was present in all plots, the number of propagules for 1 cm³ of soil varying from 24 to 226. This corresponds to 29 and 316 per gram of dry soil. The number for each plot was significantly different from the numbers for most other plots. The five sandy nurseries had, on the average, much less *Pythium* than the four on loamy soils. With a few exceptions, *Pythium* population was correlated with pH. It was not possible to establish consistent trends in *Pythium* abundance with the history of the nurseries or soil amendments applied recently. Although the soil taken from pine forest showed the lowest *Pythium* population, this was still abundant and not significantly different (at the 1% level) from the two lowest incidences in nurseries. Nursery areas established on pasture or even on previous pine forests had high *Pythium* populations. Soil amendments made no significant difference to abundance of *Pythium*.

(*b*) *Identification of Different Species*

It was not possible to recognize different *Pythium* species on the basis of growth rate or appearance of the colonies. However, most of the frequent isolates, belonging

to 11 species, eventually produced many oogonia on the plating medium and it was found possible to record the presence or absence of these species directly on each plate. Since there are no or few other studies where this has been done, and because rapid identification may greatly facilitate work on populations of this important group of fungi, it seems worth while to describe how the 11 species were identified.

TABLE 1

AVERAGE NUMBER OF PYTHIUM PROPAGULES IN SURFACE SOIL IN SOUTH AUSTRALIAN NURSERIES
Results of soil platings in a medium selective for *Pythium*

Location of Nursery	Recent Use and Amendments	Type of Soil	pH	No. of *Pythium* Colonies per 1 cm³ of Soil*	Average for Group
Bundaleer	Pine forest (still when studied)	Sandy loam	7·4	24	
Mt. Burr	Conifer seedlings	Sandy	5·2	35	79
Penola (at office)			5·7	64	
Penola I			5·2	88	
Kersbrook			6·2	91	
Penola II			6·8	118	
Caroline	Pasture previous year	Sandy	6·0	125	
Mt. Gambier (office)	Pine forest previous year	Sandy	5·0	160	
Bundaleer	Pine forest previous year	Sandy loam	6·8	140	
Wirrabara	Conifer seedlings sawdust	Sandy loam	(6·6)	90	
Wirrabara	Bark and chips Conifer seedlings	Sandy loam	(6·6)	43	
Wirrabara	Conifer seedlings	Sandy loam	6·6	52	
Tarpeena	Conifer seedlings		6·2	105	
Bundaleer	Conifer seedlings		5·9	78	152
Adelaide (Kemp's)	Ornamentals		7·0	117	
Mt. Gambier mill	Conifer seedlings	Loamy	6·5	185	
Belair	Conifer seedlings		7·4	226	
Belair	*Eucalyptus* forest previous years	Loamy	7·4	78	
Least significant difference (1% level)				30	14

* Adjusted from values for 0·07 cm³ soil, which was the actual amount of soil in each plate.

Most of the colonies belonged to *P. irregulare* Buisman or *P. mamillatum* Meurs which can be readily identified (Plate 1, Figs. 1–4). The isolates of these agreed in general with the descriptions given by Middleton (1943). An additional feature of most of these isolates of *P. irregulare* was the irregularly elongated, mostly small oogonia, which, when intercalary, were frequently orientated transversely (Plate 1, Fig. 2). Lengthwise orientation of intercalary oogonia is common in many other species, e.g. in *P. rostratum* Butler. This feature was useful especially in identifying strains that only seldom produced the few irregularly located spines, which otherwise

TABLE 2

PYTHIUM SPECIES IDENTIFIED IN SURFACE SOIL IN SOUTH AUSTRALIAN NURSERIES

Results are for 30 replicate platings of 0·07 cm³ of soil from each plot. Values in parenthesis are significant at least at 5% level (most are significant at 0·1% level). An asterisk indicates that such values are significantly different (5% level) from other values in that particular group

Location of Nursery (see Table 1)	*Pythium* Species Identified (No. out of 30 replicates):							
	mamillatum	*ultimum*[1]	*irregulare*[2]	*paroecandrum*[3]	*rostratum* Group[4]	*oligandrum* Group[5]	*echinulatum*	*col*[6]
Pine forest	—	9	6	—	15	—	3	—
Mt. Burr	26	—	11*	—	2	—	—	—
Penola (office)	7	—	29	—	—	—	—	3
Penola I	25	—	26	—	—	3	—	1
Kersbrook	25	1	30	—	5	17*	—	1
Penola II	29	—	26	—	—	—	—	—
Average for sandy soils	(22)	(0)	(23)	—	(1)	(4)	—	(1)
Caroline (pasture)	2	12	28	—	14	—	11	2
Mt. Gambier (office)	9	1	30	—	2	—	—	—
Bundaleer	—	—	28	—	—	—	1	—
Wirrabara (sawdust)	6	16	30	3	6	16	5	2
Wirrabara (bark etc.)	—	(3)	22	—	10	(4)	1	2
Wirrabara (seedlings)	—	(4)	21	4	14	(2)	—	1
Tarpeena	27	—	28	—	1	—	—	1
Bundaleer	—	—	29	3	14	6	2	—
Adelaide (Kemp's)	—	8	14*	28*	9	3	1	1
Mt. Gambier mill	1	5	30	1	3	3	—	1
Belair	9	5	30	—	—	—	—	—
Average for loamy soils	(2)	(5)	(26)	(8)	(7)	(3)	(1)	(1)
Belair (*Eucalyptus*)	7	13	30	—	6	1	3	—
Totals	173	77	442	39	101	55	27	15
Average	10	4	25	2	6	3	2	1

[1] Only strains readily producing oogonia. [2] May include a few *P. polymorphon*. [3] Atypical, close to *P. irregulare* and *P. debaryanum*.
[4] Combined with *P. iwayamai*. [5] Combined with *P. acanthicum*. [6] The code of a new species to be described elsewhere.

are so characteristic of the oogonia of *P. irregulare* (Plate 1, Fig. 2). An unexpected phenomenon was the common, sometimes prevalent production of falcate, long-stalked antheridia whenever the medium was kept under the antibiotic solution. Such an antheridium is shown in Plate 1, Figure 1. It is similar to that illustrated by Sideris (1932) for his species *P. polymorphon*. According to Middleton (1943) this species can be identified on the sole basis of these characteristic antheridia. Yet the same fungi that predominantly produced falcate antheridia in solutions or water reverted to the typical club-shaped antheridia of *P. irregulare* on drier media (1·5% cornmeal agar). This effect of water in making the antheridia falcate was also observed when several hyphal-tip cultures were again irrigated with water although the tendency varied between isolates. These two species are otherwise described as very similar, the oogonia of *P. polymorphon* also occasionally being slightly irregular and possessing a few spines (Sideris 1932; Middleton 1943). Therefore, one must conclude that some isolates of *P. irregulare* may, under certain conditions, look identical to *P. polymorphon* or that the description and identification of the latter are based on environmental modifications of *P. irregulare*. If the latter should be found true, the name *P. polymorphon* should be abandoned and the description of *P. irregulare* amended (as *Pythium* species are often examined in wet media for zoospore production). If the observed phenomenon applies only to certain local strains of *P. irregulare*, Table 2 may include some *P. polymorphon* under a wrong name. However, *P. polymorphon* could not have been very common because, in the numerous isolates examined, none had predominantly falcate antheridia on solid media, their proportion varying from 0 to 5%.

P. mamillatum was identified on the basis of its oogonia usually having numerous, fairly regular, broadly-based spines, plerotic oospores, and club-shaped monoclinous antheridia (Plate 1, Fig. 3). Contrary to Middleton (1943), all the cultures of this species from South Australia had a varying proportion, up to one out of five, of quite different oogonia, with few or no spines and a definitely aplerotic oospore (Plate 1, Fig. 4). Such oogonia resembled some of those of *P. irregulare*. However, by careful observation of a large number of oogonia it was possible to decide whether both these species were present or only *P. mamillatum*.

The species designated as *P. paroecandrum* Drechsler was not identical with the description of this species but was closer to this than any other. The isolates of this species were also close to *P. debaryanum* Hesse and to *P. irregulare*. It is possible that, especially amongst very numerous oogonia of the latter, a few of *P. paroecandrum* might have been missed or that a few of *P. debaryanum* were erroneously ascribed to *P. paroecandrum*. However, among the sample cultures there was only one typical *P. debaryanum*. A peculiarity of the isolates designated as *P. paroecandrum* was the frequent occurrence of oogonia that had one swollen sessile hypogynous antheridium and another clavate one with a fairly long monoclinous stalk (Plate 1, Fig. 5). Further studies are needed to decide whether these fungi should be described as a new species.

All the isolates identified as *P. ultimum* Trow usually had monoclinous sessile or, rarely, hypogynous or short-stalked antheridia and always clearly aplerotic oospores (Plate 1, Fig. 6). The sessile type of antheridium shown in this figure was also common in *P. rostratum* which, however, had plerotic oospores.

A taxonomic problem was also encountered with *P. rostratum*. This group included many typical isolates of this species with large plerotic oospores and mostly sessile or swollen hypogynous antheridia (Plate 1, Fig. 7); it also included a few isolates agreeing with the description of *P. iwayamai* Ito and several intermediate between these. The main difference was in the frequency of mostly monoclinous stalked, large antheridia, prevalent in *P. iwayamai* (Plate 1, Fig. 8).

P. oligandrum Drechsler (Plate 1, Fig. 9) and the somewhat rarer *P. acanthicum* Drechsler (Plate 1, Fig. 10) were typical and easy to identify by their spiny oogonia, contiguous sporangia, and the saccate antheridia of the latter. As they were not very common and were similarly distributed, they are combined in Table 2. *P. echinulatum* Matthews, though sometimes resembling *P. mamillatum* and *P. oligandrum* on account of its spiny oogonia, was identified by its peculiar antheridium (Plate 1, Fig. 11). However, several sample cultures were necessary to ascertain the presence of sporangia and thus to exclude confusion with *P. artotrogus* (Mont.) de Bary. A new species, coded as *P. col* (to be described elsewhere), was readily identified on the basis of its very thick blue oospore wall (Plate 1, Fig. 12).

Several isolates were of species that either occurred very rarely or could not be identified on the isolation plates. In spite of laborious efforts to produce both the oogonial and zoosporic stages with various means, most of these isolates remained sterile and could not be identified. There was some evidence that several of these were unusual strains of *P. irregulare*, *P. ultimum*, or *P. myriotylum* Drechsler. The remainder were a very small fraction of the isolates.

One further interesting species, identified by irregularly inflated sporangia, very small oogonia with plerotic oospores, and lack of antheridia, was coded as *P. dis* (to be described elsewhere as a new species). It was found only in the Caroline nursery which had been established recently on a grass pasture.

(c) *Occurrence of Different Species*

χ^2 tests were made to test the significance of the differences in 16 comparisons. These were chosen to illustrate the most interesting differences in pairs of plots, or of groups of plots each with similar data pooled together. All these 16 differences were significant at the 5% and most at the 0·1% level (Table 2). There were also significant differences in the horizontal direction, that is (in the frequency) between species within a plot or group of plots. For instance in most plots, *P. irregulare* was significantly more frequent than any other species. However, these were rather obvious differences and have not been marked.

Very high frequencies were found only for *P. irregulare* in 15 plots, *P. mamillatum* in five plots, and *P. paroecandrum* in one plot. In many species the frequencies differed greatly between the plots. *P. col* was unique in occurring in many plots of different soils but never frequently and *P. dis* in occurring only in one plot but in this with a high frequency (16 out of 30).

The only plot in pine forest which had the lowest total incidence of *Pythium* also had a complement of species different from other plots as is shown in Table 1. The ornamental nursery and, especially, the conifer nursery freshly established on grass

pasture also had their peculiar complements. This would have been expected because of their greatly different use or history. The five plots on sandy soils had distinctly more *P. mamillatum* and somewhat less *P. ultimum* than the four on loam. One of the plots on an intermediate soil also had many *P. mamillatum* propagules. Other significant differences were few and not consistent with any factor suggested as a possible cause. Most of the nurseries were fairly but not exactly similar in the frequency with which different *Pythium* species occurred.

IV. Discussion

The amount of soil used per plate (0·07 cm^3) was a suitable compromise for counting both rare and common *Pythium* species at once. Increasing the amount would not greatly increase the records of rare species because most of their colonies would be masked by the common and fast-growing ones such as *P. irregulare*. According to Schmitthenner (1962) the "soil particle" plate method is suitable for isolation of rare *Pythium* species.

The true numbers of *Pythium* propagules in soil were probably slightly higher than shown in Table 1. This is so because two colonies, by chance close to each other, may mingle and be counted as one, and because humus or loamy soil particles each containing *Pythium* often form small aggregates (Warcup 1952; Schmitthenner 1962). The true populations are thus higher, especially in loamy soils or those with a high organic content. For assessing most accurately small differences in the frequency of common species, the amount of soil per plate should be smaller.

The result of outstanding importance was the generally high density of *Pythium* population in spite of low disease incidence in several of the plots and regardless of the soil type, history, or amendments. A seedling can hardly avoid contact with *Pythium* propagules if every cubic centimeter of surface soil contains more than 35 of them, many of them being *P. irregulare*, *P. ultimum*, and *P. mamillatum*, which are pathogenic to pine seedlings. The rest of the isolated species may also be pathogenic to pine seedlings. Such an abundance of pathogens supports the hypothesis that the damping-off incidence in these nurseries is mainly dependent on factors other than *Pythium* populations in soil. Warcup (1952) has mentioned an instance of little damping-off disease in a conifer nursery with high *Pythium* density. Griffin (1958) has given evidence indicating that low pH decreases conifer damping-off through increased resistance of the seedlings.

There is obviously a lower and an upper limit outside which the abundance of a pathogen must strongly affect disease. Vaartaja, Cram, and Morgan (1961) found that in experimental damping-off of conifers, the disease incidence largely depended on environmental factors if the inoculum was light; if this was very heavy, environment made little difference and even unusual pathogens were capable of causing high disease incidences. One of the tasks of pathological research is to find these limits for various diseases. Kerr (1963) has recently approached this in a study of a root rot–wilt complex of peas in South Australia. This disease was reproduced artificially when the number of pathogens in virgin soil was increased by controlled inoculation to the levels in "sick" soils. This meant increase of *Pythium* populations from the level of 16 to 97 per gram (dry soil) in a sandy soil and from 26 to 343 in

loamy sand. This is about the same range as found in the forest nurseries above (Table 1). However, after harvesting peas in a greenhouse, numbers as high as 870 per gram were recorded. Schmitthenner (1962), using thoroughly tested techniques, reported *Pythium* numbers from 245 to 635 per gram in seven heavy soils in Ohio. Warcup (1952), without selective media, found 55–244 per gram in three sections of an old forest nursery in England; the range in another nursery was 595–687 per gram; the numbers in five other nurseries were not given in detail but were reported as comparable with these. Angell (1945) found, after sieving and sedimentation for 4 weeks (to avoid bacteria), numbers of the order of 1000 *Pythium* propagules per gram of surface soil in Australia.

Warcup (1952) found no *Pythium* propagules in pure pine forests and only few in mixed conifer–hardwood stands. This is a contrast to the result here in Tables 1 and 2. It must be added, however, that the abundance of *Penicillium* and other antagonists in the pine soil studied was enormous, and the high *Pythium* number could not have been found without the use of strongly selective media.

As is seen from Table 2 and from the data of Warcup (1952) and Schmitthenner (1962), the proportion of the different *Pythium* species probably varies between soils more than does the total incidence. However, the species found most commonly in soils appear largely the same (*P. irregulare*, *P. ultimum*, *P. rostratum*, *P. mamillatum*, *P. oligandrum*) in different parts of the world (Table 2; Meredith 1940; Middleton 1943; Warcup 1952; Miller, Giddens, and Foster 1957; Schmitthenner 1962). The new species *P. col* and *P. dis*, found in South Australia, have conspicuous characteristics and probably would have been recorded if they were common in England or the United States of America. Similarly *P. iwayamai* (Table 2) may have a restricted distribution (Middleton 1943). *P. echinulatum* may also be more common in South Australia (Table 2) than elsewhere (Middleton 1943). On the other hand, *P. monospermum* Pringsh., *P. conidiophorum* Jokl, and *P. aphanidermatum* (Edson) Fitzp., commonly found in certain Ohio soils by Schmitthenner (1962), were not encountered during this study. *P. intermedium* de Bary, reported by Warcup (1952) in English nursery soils, was not isolated during this study but was found in diseased seedlings in one of the nurseries.

V. References

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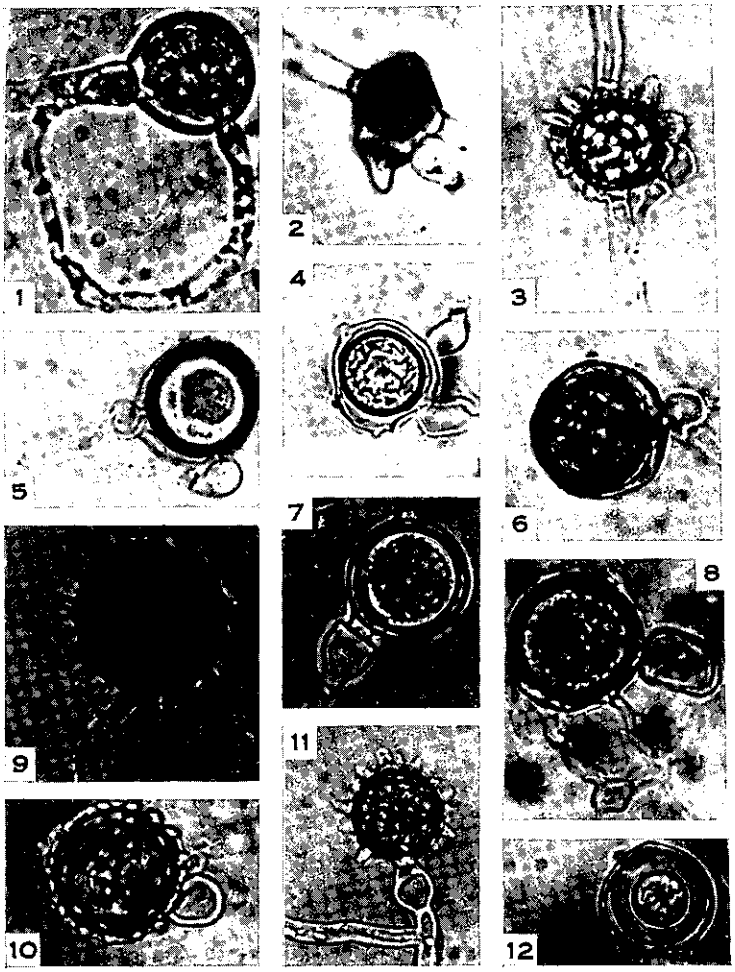
PYTHIUM SPECIES IN NURSERY SOILS



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EXPLANATION OF PLATE 1

All figures × 700

Fig. 1.—Oogonium of *P. irregulare* with the falcate antheridium typical of *P. polymorphon* but produced by *P. irregulare* in liquid media.

Fig. 2.—Typical oogonium of *P. irregulare* on dry media.

Fig. 3.—Typical oogonium of *P. mamillatum*.

Fig. 4.—Aplerotic type of oogonium of *P. mamillatum*.

Fig. 5.—Oogonium of a *Pythium* tentatively identified as *P. paroecandrum*.

Fig. 6.—Oogonium of *P. ultimum* with the typical sessile antheridium.

Fig. 7.—Oogonium of *P. rostratum* with a hypogynous antheridium.

Fig. 8.—Oogonium of a typical *P. iwayamai*.

Fig. 9.—Oogonium of *P. oligandrum*, typically without antheridia.

Fig. 10.—Oogonium of *P. acanthicum*.

Fig. 11.—Oogonium of *P. echinulatum* with a typical antheridium.

Fig. 12.—Oogonium of *P. col* (code for a new species to be described).