Responses of Pythium ultimum and Other Fungi to a Soil Extract Containing an Inhibitor with Low Molecular Weight

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ABSTRACT

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In 1971, samples of rain waters that percolated through clay loam in a lysimeter were collected, filtered (0.2 μm pore size), and bioassayed for growth response effects on mycelia of *Pythium ultimum*. Growth (radial colony extension) varied from 25 to 160% of that in distilled water controls. Following and during regularly spaced heavy rains, 23 August to 29 September, there were consistent inhibitory effects (growth 25-68%). Bioassays after ultrafiltration (Amicon) and gel chromatography (Sephadex, Bio-Gel) indicated the presence of many inhibitory substances with molecular weights (MW) that varied widely (<3,000, 6,500, 7,000, 18,000, and 33,000) before and after those dates. From 23 August to 8 September very strong inhibition was caused by an unidentified substance with MW about 150. After 8 months of storage at 5 C and after being boiled for 5 minutes in open containers, the extract that contained this substance remained inhibitory, with only a slight decrease in potency. The in vitro effects of a sample collected on 31 August were studied on 42 soil fungi. The response of different groups of fungi varied significantly ($P = 0.001$) and their descending order of sensitivity was: (i) soil basidiomycetes; (ii) *Pythium* spp.; (iii) miscellaneous soil fungi; (iv) *Fusarium* and *Cylindrocarpon* spp.; and (v) *Penicillium* and *Gliocladium* spp. The inhibitory effects were reduced significantly by amending the extract with 5% sucrose and 0.5% yeast extract.

Additional key words: fungistasis, *Thanatephorus practicola*.

In several tests, extracts from a sandy nursery soil were more inhibitory to *Pythium ultimum* Trow and to *P. irregulare* Buism. than to *Thanatephorus practicola* (Kotila) Flentje (10). *Pythium ultimum* also was inhibited to a greater extent than was *Fusarium oxysporum* (Schlecht.) emend. Snyder & Hans. by further extracts from the same soil (1). Both extracts remained inhibitory after heating, suggesting the presence of nonvolatile heat-stable inhibitory substances. A third study (9) with extracts from two soils indicated differences among ten fungi. Thus, most *Pythium* spp. were inhibited but *Cenoccoccum graniforme* (Sow.) Ferd. & Jørg. and *Gliocladium fimbriatus* Gilman & Abbot were not. *Fusarium* spp. often have been found to be tolerant to soil fungistasis (2, 3, 4, 5, 6, 7, 8, 11, 13) and some data suggest that *Thanatephorus* spp. also are fairly tolerant (6, 11).

Until recently, factors causing and modifying soil fungistasis were poorly understood (1, 10, 12, 13) but now it is possible to group them as follows: (i) competition for nutrients which is particularly important in germination inhibition of small fungal propagules (3, 7) [nutrients also may act as "stimulants" (12) by counteracting inhibitors (1, 8, 10)]; (ii) volatile inhibitors (4, 12) which are best detected by culturing test organisms in an enclosed container with the soil; and (iii) nonvolatile inhibitors [separable from soil in aqueous or organic solvent extracts (1, 6, 8, 10, 11, 13)]. Extracts with stable inhibitors can be fractionated with such methods as molecular sieving chromatography or electrophoresis (8, 10).

Watson and Ford (12) and Vaartaja and others (1, 8, 10) emphasized that soil mycostasis seems to be caused by more than one mechanism and that it commonly is modified by stimulants. Consideration of the existence of the latter is essential in understanding the variation and labile balance probably prevailing in many fungistases.

In this study, the responses of different types of soil fungi to one particularly interesting soil extract were examined. This extract was selected after a consistent inhibition occurred in a test series with *P. ultimum*. Attempts were made to characterize inhibitors found in several extracts. To test for the postulated counteraction by stimulants, growth responses were recorded when the extract had been amended with sucrose and yeast extract. Such amendment earlier (1, 8, 10) counteracted the effects of similar inhibitors of *Pythium* and *Thanatephorus* species.

MATERIALS AND METHODS

Soil extracts and bioassays.—A 5-cm layer of clay loam soil from Ramsayville, Ontario, Canada, pH approximately 6.0, with its herb, grass, and moss vegetation intact, was transferred to a lysimeter. As described earlier (8), water percolating through the soil was collected after rains and bioassayed (1) with *P. ultimum* isolate 9248. Earlier, this isolate as well as four other pathogenic *Pythium* spp. were inhibited by extracts from two Ontario soils (9).

Extracts obtained from this soil were prefiltered through glass wool and passed through Amicon "ultrafilters" with nominal "cut-off" points at molecular weights 100,000 (XM-100), 300,000 (XM-300) or 10,000 (PM-10). Fractions from these filtrations usually were refiltered through 0.2 μm Sartorius filters and then bioassayed. Radial extension of *P. ultimum* colonies in 20 hours was measured on a medium consisting of an extract, or a fraction thereof, and purified 2% agar (1:1, v/v). The growth was compared with that on control agar prepared with distilled water instead of soil extract. Growth of 0-90% (of controls) in extract bioassays, indicated full or partial inhibition and 110%, or more, stimulation (8). Only radial extension growth was studied as it was necessary to make many measurements quickly and because extension growth seems to be particularly important for many soil-borne pathogens (8). Inhibitors may act mainly during the phase of saprophytic extension growth before infection of the plant.

Strongly inhibitory extracts or the ultrafiltrates were fractionated by gel filtration ("molecular sieving") with Sephadex G-15, Sephadex G-75, or Bio-Gel P-30. Small molecules entering the gel matrix are delayed in inverse proportion to their sizes. This was used to estimate the molecular weights (MW) of the inhibitors present in the extracts (8). "Blue Dextran" and cobalt chloride were used as markers in calibrating the molecular fractions for MW $>$ 100,000 and MW 130. Because of random but considerable effects of the gel itself, only the growth percentages of 0 to 65 or $>$ 123 were considered to indicate significantly ($P = 0.01$) active gel fractions (8).

The fungi.—One strongly inhibitory filtrate was stored at 5 C and assayed, following the above procedure, with a variety of fungi obtained from forest and nursery soils in Ontario. The fungi were grouped as follows: (i) Soil basidiomycetes including those identified as *Agaricus silvicola* (Vitt.) Sacc., *Thelephora terrestris* (Ehr.) Fr., *Lepista nuda* (Bull. ex Fr.) Cooke, *Macrolepiota procera* (Scop. ex Fr.) Sing., and *Waitea circinata* Warcup & Talbot; (ii) *Pythium* spp. including *P. irregulare* Buism., *P. mamillatum* Meurs, *P. rostratum* Butler, *P. oligandrum* Drechsler, and *P. ultimum;* (iii) *Fusarium* and *Cylindrocarpon* spp. including *F. oxysporum* (Lk.) em. Snyder & Hans., *F. solani* (Mart.) Appel & Wr., and *Cylindrocarpon destructans* (Zins.) Sholten; (iv) *Penicillium* spp., *P. frequentans* Westl., *P. vermiculatum* Dangeard and *Gliocladium roseum* Bain., and *G. fimbriatum;* and (v) Other miscellaneous soil fungi including *Alternaria alternata* (Fr.) Keissler, *Gliocladium virens* Miller, Gid. & Foster, *Trichoderma hamatum* (Bon.) Bain., *Chaetomium globosum* Kunze, and *Mortierella elongata* Linn. Each group consisted of seven isolates, some not identified to the species.

In addition to the 35 fungi included in the five groups, five other species also were tested. These were: *Thanatephorus praticola* (Kotila) Flentje (a well-studied pathogen); *Cenococcum graniforme* (Sow.) Ferd. & Jørg. (a common mycorrhizal symbiont on various coniferous and hardwood trees); *Gyrodon merulioides* (Schw.) Sing. (a mycorrhizal symbiont on *Fraxinus* spp.); *Zygorhynchus moelleri* Vuill. (an extremely rapid common colonizer of nursery soils); and three isolates of *Pythium sylvaticum* Campbell & Hendrix including + and − mating type heterothallic strains and a homothallic strain (virulent pathogen common in North America and Europe).

Most of these fungi were included in a four-way test of a soil extract: (i) XM-300 filtrate from the extract collected 31 August 1971 and stored at 5 C for 8 months; (ii) as for i but the inhibitory action weakened by about 1:1 dilution with distilled water and by an additional storage for 2 weeks, mostly at 5 C and partly 25 C; (iii) as for i but amended with 5% sucrose and 0.5% yeast extract when tested; (iv) as for ii but amended as for iii. The bioassay results with the five groups of fungi and four extract treatments were subjected to an analysis of variance. The significances of the differences of the means were tested by the Student-Newman-Keuls method.

RESULTS

Inhibition of Pythium ultimum by the soil extracts.—*Seasonal variation*.—There were seasonal and irregular variations in extracts from the clay soil in the lysimeter. The sample collected 3 May 1971 stimulated the growth of *P. ultimum* in the bioassays to 160% of the distilled water controls after it had been passed through a 0.22 μm filter. With the 25 May sample the growth was 100% and then varied between 58 and 134% in five samplings.

After a dry period there was a 5-cm rainfall on 10 August and the soil remained moist from six rains, each of about 2.5 cm and at 1-week intervals. Possibly as a result of the rains, the 23 August to 29 September samplings consistently showed inhibition, the growth varying between 25 and 68% of the controls in 13 samplings.

In the last three extracts in October the inhibition was weak (growth 75-93% of the controls). Another clay soil

TABLE 1. Estimation of molecular weights (MW) of inhibitory fractions of extracts from a clay soil after ultrafiltration (UF) and gel chromatography (GC)

Date of extraction 1971	MW (procedure[a], and no. of replications in parentheses)
16 August	<10,000 (UF, 2); 32,000 (UF + GC, 2)
26 August	<3,000, 6,500, 18,000, 33,000 (UF + GC, 2)
30 August	<10,000 (UF, 2) (also stimulation: 35,000, GC); ~150 (UF + GC, 3)
31 August	~150 (UF + GC, 2)
8 September	~150 (UF + GC, 2)
9 September	<3,000, 7,000 (UF + GC, 2)

[a]Amicon XM-200 or PM-10 ultrafilters; and/or Sephadex G-15, G-75 gels, or Bio-Gel P-30 gels.

exhibited the same seasonal pattern (growth 25-100% of the controls in 19 samplings) whereas an extract from a sandy soil was inhibitory only four times in 16 samplings and the inhibitions were not strong (growth 65-138% of the controls). During the strongest inhibition all three soils were at times waterlogged, but not seriously enough to damage the plants in the lysimeters.

Molecular weights.—Extract collected on 16 August from clay soil gave interesting results. When it was passed through a PM-10 ultrafilter (cut-off point MW = 10,000) both the filtrate and the retentate were inhibitory to *P. ultimum* (growth 31 and 64% of the controls). When the retentate was sieved through Bio-Gel P-30, three adjacent fractions were inhibitory (MW $\simeq$ 33,000). The MW for the inhibitor(s) in the filtrate was not determined but they must have been < 10,000. The MW estimates shown in Table 1 were made using similar procedures. Results indicating MW < 10,000 probably were associated mainly with the inhibitor of MW 150 (Fig. 1), but the particular gel (Sephadex G-75) that was used was unable to separate molecules smaller than MW 10,000. The data of Fig. 1 indicated an inhibitor in fraction 9 in each of five tests but not in any other fraction.

The inhibitor of MW 150 was demonstrated only when the gel was swelled with distilled water but not when it was swelled with acid (pH 5-7) buffers. Measurements with an electrode, placed with a small amount of water in chopped bioassay agar, gave pH 7.0-7.3 for the inhibitory fraction and 6.3-7.1 for other fractions. However, the inhibition did not seem to be a direct pH effect because *P. ultimum* grew well in agar buffered to this pH range with KOH and KH_2PO_4.

Effects of treatments on the inhibitor extracted 30 and 31 August 1971.—1) Storage.—When PM-10 ultrafiltrate from 30 August sampling was bioassayed with *P. ultimum* after two periods of storage at 5 C, the growth (percentages of distilled water controls) increased from 0 to 18% in 1 month and to 21% in 8 months. When XM-100 ultrafiltrate from 31 August sampling was bioassayed, the percentages were: fresh-41%, 8 months-40%, 9 months-25%.

2) Counteraction.—When yeast extract (0.5%) and sucrose (5%) were added to the bioassay medium, inhibition was reduced. Growth with the ultrafiltrate from 30 August sampling increased from 0 to 100%, and from 31 August sampling from 41 to 89%. These and other similar effects of nutrients were interpreted as counteraction of inhibition by the nutrients.

3) Heating.—When PM-10 ultrafiltrate from 30 August sampling was heated in an open beaker and boiled for 5 minutes, the growth of *P. ultimum* increased from 0 to 37% of controls. Such relative tolerance of heating was demonstrated for nine extracts from the same soil and suggested that the inhibitor was a nonvolatile compound.

4) Dilution.—When a 0.1 μm filtrate from 31 August sampling was bioassayed with *P. ultimum,* the growth was 35% of the controls. Dilutions to one-half or one-fifth increased the growth to 40% or 45%. Similar results were obtained for the filtrate from 8 September sampling: one-tenth undiluted = 58%, one-fifth = 75%, and one-twentieth = 95%, indicating the presence of a potent fungal inhibitor.

Effects of one extract (31 August 1971) on other fungi.—*Growth.*—As shown above, *P. ultimum* bioassays of extracts collected during the late summer 1971 from a clay loam soil indicated a consistent and strong inhibition due mainly to a relatively stable nonvolatile inhibitor with estimated MW $\simeq$ 150 (Fig. 1). The ultrafiltrate (XM-300) from 31 August sampling contained this inhibitor and its effects on different fungi are shown in Table 2 and 3. The data on the modified (stored and diluted) extracts are not given. As expected, these treatments reduced inhibition, the average effect being 22%. Analysis of variance of the data for 35 fungi of

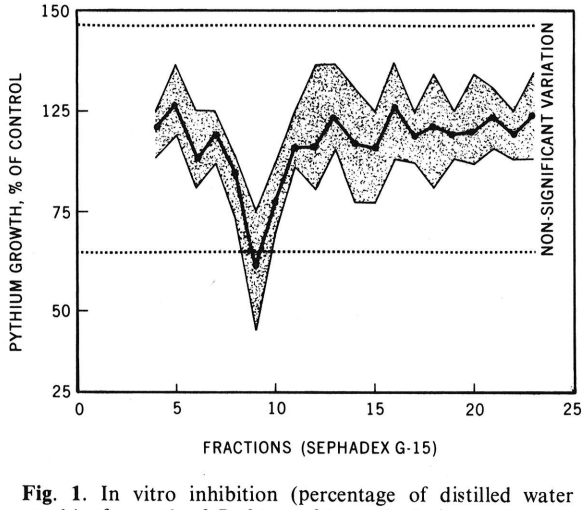

Fig. 1. In vitro inhibition (percentage of distilled water controls) of growth of *Pythium ultimum* by lysimeter extracts (taken 8 August 1971) from a clay soil. Samples were subjected to ultrafiltration (Amicon PM-10) and fractioned by molecular sieving (Sephadex G-15). Heavy line represents the averages of five sievings; the shaded area represents the variation. Fraction 9 contained the only significantly inhibitory compound(s) which were estimated to have molecular weights of ~150.

TABLE 2. Effects of an ultrafiltered (XM-300) clay soil extract (31 August 1971), before and after amendment[a], on the radial colony extension of five groups of fungi

Fungus group	Growth (% of distilled water controls)		
	Extract	Amended extract	Ave.[c]
Basidiomycetes	21	37	29
Pythium species	50	79	65
Miscellaneous soil fungi	55	98	77
Fusarium and *Cylindrocarpon* species	86	131	109
Penicillium species[b]	99	114	107
Average[c]	62	92	77

[a]Amendments were with yeast extract (0.5%) and sucrose (5%).

[b]The average included another genus, see text.

[c]With the exception of *Fusarium* vs. *Penicillium,* all the averages in the last column were different, and all the averages in the bottom line were different (P = 0.001) by the Student-Newman-Keuls test).

Table 2 indicated highly significant ($P = 0.001$) effects of the four extract treatments and of the five groups of fungi.

Although some details of the data presented in Tables 2 and 3 suggested the possibility of a treatments × fungi interaction, the overall interaction was not significant ($P = 0.01$). However, individual species seemed to differ in sensitivity to the inhibition and to counteraction of inhibition (Table 3).

Other responses.—Thanatephorus praticola hyphae showed abundant lysis earlier (in 6 days) with unamended extracts than in controls (in 10 days) or with amended extracts (in 12 days). The fungus produced basidiospores in the nonamended extracts and less abundantly in controls but not in the presence of amendments. Many fungi formed denser colonies in the presence of amendments. Where the amendment did not increase radial extension (*G. fimbriatum,* Table 3), total growth nevertheless may have increased. Sporulation of *Z. moelleri, G. virens, P. rostratum* (sporangia), and *P. irregulare* (sporangia) was markedly reduced; whereas that of *P. rostratum* (oogonia), *P. oligandrum* (sporangia), and *F. oxysporum* (microconidia only) was increased by the extract.

DISCUSSION

Demonstration of fungal inhibitors in molecular fractions of soil extracts is a unique finding reported before only in my earlier study (8) where only one fungus was tested. Bioassays of such fractions here with *Pythium ultimum* indicated that inhibitors contribute to soil fungistasis and thus agree with other recent data (1, 8, 10, 11). As discussed earlier (1, 8, 10) the kind of inhibition described here cannot be explained as lack of nutrients. For instance, the fact that dilution of the extract with distilled water decreased the inhibition is contrary to what would be expected if the inhibition were due to lack of nutrients.

TABLE 3. Examples of a filtered (XM-300) clay soil extract (31 August 1971), before and after amendment[a], on radial colony extension of individual fungal species

Fungus	Growth (% of distilled water controls)	
	Extract	Amended extract
Cortinarius sp.	3	67
Agaricus silvicola	78	60
Pythium rostratum	56	100
P. irregulare	27	82
Alternaria alternata	78	82
Trichoderma hamatum	17	75
Fusarium solani	109	160
F. oxysporum	90	123
Gliocladium fimbriatum	130	120
Penicillium janthinellum	38	85
Zygorhynchus moelleri[b]	8	95
Gyrodon merulioides[b]	14	67
Pythium sylvaticum[b,c]	39	69
Thanatephorus praticola[b]	46	159
Cenococcum graniforme[b]	50	89

[a]Amendments were with yeast extract (0.5%) and sucrose (5%).

[b]Excluded from the statistical analysis of Table 2.

[c]The three isolates of this species responded similarly.

Gel filtration revealed a strong inhibitor from a clay soil, a nonvolatile substance with an estimated MW about 150 (Table 1, Fig. 1). The inhibitor was only partly destroyed by heat as found earlier for extracts (MW unknown) from sandy soils (1, 10, 11). In addition, it withstood storage at low temperature for several months without loss of activity. Considerable variation in growth response to the extract was found among many fungi (Table 2, 3). Such variation in sensitivity to an inhibitor from soil is remarkable when contrasted to most data on soil fungistasis in the literature (3, 12, 13). Many reports show that nearly all small spores of many fungi are strongly inhibited in most soils. Some groups (e.g., the basidiomycetes) have been studied very little. The data of this study concern only one kind of factor in fungistasis, namely the extractable inhibitors, and are limited to one period of observation. It is not yet known how widely the results can be generalized. One difficulty is that the inhibitors in soil may be more important than this study indicates because of the following factors: (i) absorption to filters; (ii) dilution in chromatography; (iii) dilution in bioassays; and (iv) decomposition. Furthermore, the inhibitors may be important in certain microsites but undetectable after dilution and counteraction by stimulants in bulk extractions.

The radial extension of *Gliocladium fimbriatum* was stimulated by an extract that inhibited most fungi which is in agreement with earlier data (9). The tolerance of *Fusarium* and related spp. was noted earlier (1, 2, 3, 5, 6, 7, 11, 13). *Fusarium solani,* however, is fairly sensitive (13), particularly to volatile inhibitors (4).

The soil basidiomycetes, as a group, were sensitive to the inhibitor. However, not all members of this heterogeneous group responded similarly and, therefore, more study is needed to ascertain variation within the group. *Ceratobasidium cornigerum* (Bourd.) Reg. seemed to be tolerant to, or even stimulated by, extracts that were inhibitory to many other fungi (9). *Penicillium* spp. were tolerant with the exception of *P. janthinellum.* Dix (2) found this species to be fairly tolerant of competition in the rhizosphere of bean. However, the causes of fungistasis in Dix's study appear to be different from those reported herein; his soil extracts were not inhibitory after passage through a Seitz filter.

It was surprising that the rapid soil colonizers *Z. moelleri, T. hamatum,* and *G. virens* were strongly inhibited. On the other hand, yeast extract and sucrose had a strong counteractive effect on their inhibition, particularly on that of *Z. moelleri.* Counteraction of the inhibition by nutrients (Table 2, 3) was noted for most fungi tested. Yeast extract and sucrose amendments failed to increase growth of only a few fungi, mainly those not greatly inhibited by the extract. The fact that the fraction of MW 150 was inhibitory to *P. ultimum,* when obtained with distilled water as the eluant, but not with acid buffers, indicates a possible interaction of a different mechanism. The chemical characterization of this fraction remained preliminary and will not be reported until more material becomes available. The inhibitions in 1972-1975 were not consistent or strong enough for this. The occurrence of the inhibitor(s) with MW $\simeq$ 150 thus

may be limited to certain microbiological situations depending on coincidences of weather as described above.

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