Soil Suppressiveness to a Plant Pathogenic *Pythium* species

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ABSTRACT

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Attempts to induce soil suppressiveness to a plant pathogenic *Pythium* sp. by adding mycoparasitic *Trichoderma* spp. (previously reported to induce suppressiveness to *Rhizoctonia solani*) were not successful. However, weekly addition of dried ground bean leaf material to Nunn sandy loam soil (pH 7.3 at 26 C) at weekly intervals over a 6-wk period decreased propagule population density of the plant pathogenic *Pythium* sp. Suppressiveness was not observed in otherwise similarly treated soil incubated at 19 C or adjusted to pH 5.0. The decline in propagule population density of the plant pathogenic *Pythium* sp. was associated with an increase in population density of an unidentified mycoparasitic species of *Pythium*. The latter induced early lysis of germinating sporangia of the plant pathogenic *Pythium* sp. in vitro and on membrane filters laid on soil infested with the mycoparasitic isolates. Addition of the antagonist to soil suppressed the competitive saprophytic ability of the plant pathogenic *Pythium* sp. and also its capacity to induce preemergence damping-off of cucumber. Therefore, it induced both pathogen and disease suppressiveness. Since the antagonist was not pathogenic to plants commonly used in agriculture, it has potential for use as a biological control agent against diseases induced by plant pathogenic species of *Pythium*.

Plant pathogenic species of *Pythium* have a broad host range and are widely distributed. They cause pre- and postemergence damping-off and root rots and considerable yield losses in many important crops. Fungicides currently are used to control these diseases; however, control is not always effective, and the intensive use of fungicides increases environmental pollution, health hazards, and sometimes induces phytotoxicity. To reduce the deleterious effects of fungicide application, alternative control methods are required. It is, therefore, important to develop economically feasible biological control procedures for use in agricultural production.

Induction of soil suppressiveness to a plant pathogen may provide long-term plant protection. Repeated plantings of a susceptible host in raw soil induced the soil to become suppressive to *Rhizoctonia solani* (6,15,24). It was suggested that replantings induced germination of the pathogen's resting structures and enhanced parasitism of its hyphae by mycoparasitic *Trichoderma* spp. Indeed, increase in population densities of *Trichoderma* spp. in soil during monoculture and increase in suppressiveness was followed by a drastic decline in inoculum densities of *R. solani*.

In the present study, attempts were made to induce soil suppressiveness to a plant pathogenic *Pythium* sp. by promoting saprophytic development of the pathogen in natural soil similar to that induced to *R. solani* in monoculture of radish (6,24). Since suppressiveness in monoculture of radish was mediated by *Trichoderma harzianum* (24), the objective of the initial experiments was to determine the influence of such antagonism on development and activity of *Pythium*.

MATERIALS AND METHODS

Soil. Nunn sandy loam was used in all experiments. Soil characteristics were as follows: soil batch B (pH 6.8; collected in July 1982) or soil batch A (pH 7.3; collected in April 1983); <1% lime; conductivity, 0.4 mmhos/cm; organic matter, 1.1%; and 1 mg of nitrate N, 9 μg of extractable Zn, and 3.2 μg of extractable Fe per gram of soil. Soil moisture characteristics were described previously (11). Soil was collected from the vicinity of Nunn, CO, air-dried, sieved through a 4-mm-mesh screen, and stored in covered containers before use. These batches of soil contained an indigenous population of a species of *Pythium* (hereafter designated isolate N1) that induced preemergence damping-off of at least eight plant species.

Isolates of *Trichoderma* spp. Cultures of *Trichoderma harzianum* T12 and *T. koningii* Rifai T8 isolated from a New York soil were provided by G. E. Harman (New York State Agricultural Experiment Station, Geneva 14456). *T. harzianum* T95 is a benomyl-tolerant mutant that was derived from an isolate effective in inducing soil suppressiveness to *R. solani* (7).

Saprophytic development of *Pythium* spp. in soil. Nunn sandy loam soil was used either at its original pH (pH 7.3) or was acidified to pH 5.0 with 1 N H_2SO_4 (1 ml of 4% solution per gram of air-dried soil). During the period of experimentation, the adjusted pH did not vary more than ± 1 pH unit from the original. Dry, mature green bean (*Phaseolus vulgaris* L. 'Pinto') leaves (ground and sieved through a 1-mm-mesh screen) were incorporated into the soil at 0.3 g of leaves in 100 g of soil. Separate batches of the soil were watered to −0.3 bars (15%, w/v) with either a conidia suspension of each of the three isolates of *Trichoderma* (final concentration 10^7 conidia per gram of soil) or with sterile tap water and thoroughly mixed. Soils were placed in 6 cm (diameter) × 9 cm glass jars (170 grams per jar, three jars per treatment, and completely randomized) and covered with polyethylene sheets to reduce evaporation. The jars containing soil were incubated at 19 ± 1 C or 26 ± 1 C for 7 days. At the end of the incubation period, the soils were air-dried, ground, and mixed thoroughly. The soils were amended repeatedly and treated in this manner for a total of six 1-week incubation cycles. During the experimental period, soil samples from each jar were tested to determine propagule density of *Pythium* spp. by the soil drop method (37) and by counting colony-forming units (cfu) on dilution plates of a selective medium (31). Population density of *Trichoderma* spp. was determined on dilution plates of a selective medium (12).

In vitro hyphal interaction among isolates of *Pythium* spp. Cellophane membranes were washed in boiling distilled water, autoclaved, and placed on the surface of water agar. Water agar disks, with mycelium of *Pythium* spp. isolates growing on them were placed at the opposite ends of the cellophane membrane. After 3 days of incubation at 26 C, the cellophane membranes were removed at the interaction areas. Interactions were observed under a light microscope at ×200 and ×400.

Pathogenicity test for isolates of *Pythium* spp. Cultures of *Pythium* spp. isolates were grown on potato-dextrose agar for 5 days at 26 C. Agar disks (8-mm diameter) with mycelium, or sterile medium as controls, were placed in autoclaved soil at a depth of 1 cm. Five seeds of each of the various plants were placed directly on the agar disks in each of four pots per host under conditions previously described (6,14,15). Soils were watered to −0.3 bars (15%, w/v), covered with transparent mylar sheets, and incubated at 19 C (two pots of each treatment) or at 26 C (two pots of each treatment). Counts of healthy seedlings were made after 14 days.

Generation of high population densities of isolates N2 and N3 of *Pythium* sp. In preliminary trials it was not possible to increase propagule densities of two isolates of a *Pythium* sp. (isolated from Nunn soil and hereafter designated as isolates N2 and N3) by conventional pure culture techniques or on autoclaved seeds. Therefore, propagule units were generated by the following method. Isolates N2 and N3 of *Pythium* were grown on a rolled oats and water medium (13) at 26 C for 14 days. Mycelial mats resulting from the cultures were removed and placed in previously autoclaved soil amended with bean leaf meal (0.3 g/100 g of soil). Soil moisture was adjusted to −0.3 bar with sterile tap water. The soils were placed in containers and incubated for 7 days at 26 C. No attempt was made to maintain an axenic culture; therefore, the *Pythium* sp. developed in ecological conditions roughly parallel to those found in natural soil. At the end of the incubation period, the infested soils were air-dried, ground, and sieved (1-mm-mesh screen). Counts of cfu (∼10^4 cfu/g) of isolates N2 and N3 were determined by plate dilution on a selective medium (31).

Testing the effect of isolates N2 and N3 of *Pythium* sp. on the saprophytic development of *Pythium* sp. isolate N1. Propagules of isolates N2 or N3 were mixed in the Nunn sandy loam soil. Population densities of isolates N2 and N3 were estimated as ∼100 cfu/g of soil by plate dilution on a selective medium. This mixture was incubated 2 wk at 26 C and amended with bean leaf meal (0.3 g/100 g of soil) at 0 and 7 days after the beginning of the experiment. Population densities of isolates N1, N2, and N3 were monitored at 0, 7, and 14 days after the initiation of the experiment.

Assay for sporangial germination, hyphal lysis, and formation of new sporangia by isolate N1 on soil. The soil (batch A, pH 7.3) was incubated at 26 C and −0.3 bar moisture with six weekly additions of ground dried bean leaves (0.3%, w/w) and air-dried. An unamended soil served as a control. Ten-gram samples from each soil were mixed with 30 mg of bean leaf meal and placed in 45-mm-diameter plastic plates. The soil in each plate was mixed with 3.0 ml of sterile deionized water and the soil surface was smoothed with a spatula.

Sporangia of isolate N1 harvested from 1-wk-old oatmeal and water cultures were washed three times in sterile deionized water and resuspended to give 3×10^3 sporangia per milliliter. Samples of 0.3 ml of suspension were placed on 26-mm-diameter Nuclepore membranes (Nuclepore Corp., 7035 Commerce Circle, Pleasanton, CA 94566) on a Millipore holder. The water was removed through the membrane with suction and the membranes were placed on the smooth soil surface (see preceding paragraph). The plates were covered, placed in polyethylene bags, and incubated at 26 C for 2, 24, 48, or 72 hr. After incubation, the membranes were removed from soil, placed in 0.05% aqueous cotton blue for 1 min, and prepared for microscopic observation either by the method described by Sneh (35) or by Bristow and Lockwood (3). All sporangia on each membrane were counted.

Autoclaved soil amended ith 0.3% dried bean leaf meal was incubated with isolates N1, N2, or N3. After 2 wk of incubation, the soils were dried (population densities of each was ∼10^4 cfu/g of soil). The soils were treated as described above to test the effect of the isolates in axenic culture in soil on germination, lysis, and the formation of new sporangia by isolate N1. Two membranes for each treatment were used for each of three trials.

Assay for disease incidence in soils infested with isolates N2 or N3 of *Pythium* sp. Isolates N2 or N3 of *Pythium* sp. were added to soil batches A and B, respectively (nontreated soil served as a control). Water was added as previously described, and the soils were incubated for 2 wk. Soils were amended with two weekly additions of dried bean leaf. These were diluted at ratios of 1:9 (90%) and 1:99 (99%) in raw soil (batch A). Two hundred-gram samples were placed in eight plastic pots (11 cm in diameter × 8 cm). Six cucumber (*Cucumis sativus* L. 'Straight Eight') seeds were placed in each pot (five pots per treatment). Soil moisture was adjusted to −0.3 bar. The pots were covered with mylar sheets and incubated at 26 C. Numbers of healthy seedlings were recorded after 7 days. Statistical analyses were performed by using Fisher's least significant difference test as recommended by Madden et al (27).

RESULTS

Isolation of *Pythium* spp. from the Nunn sandy loam soil. *Pythium* spp. were enriched in samples of soils A and B by procedures described elsewhere (22). In both soils, isolates from hyphal tips of germinating hyphae resembling *P. ultimum* Trow (sensu Stanghellini and Hancock [37]), were grown on oatmeal agar or V8 broth at 21 C for 14 days in darkness. All the isolates formed spherical, mostly acrogenous sporangia (∼21 μm in diameter); however, no oospores were observed. A typical isolate (designated N1) had an arachnoid habit of growth on potato-dextrose agar. Sporangia germinated only by germ tubes within 45–90 min at 26 C. The optimal temperature for radial growth and sporangial germination on potato-dextrose agar was between 25 and 30 C, while optimal temperature for saprophytic development in soil (assayed as in the procedure described by Liftshitz and Hancock [23]) was between 16 and 20 C. The possibility that the isolate was a heterothallic *Pythium sylvaticum* was tested by growing it in dual cultures with each mating type of *P. sylvaticum* (ATCC 18195 and ATCC 18196). No oospores were formed. Such asexual sphaerosporangial pythia with morphology similar to *P. ultimum* are found commonly in the United States. For example, they could be members of the SP1, 5, 9, and 10 groups described by Schmitthenner (34). Preczanka and Abawi (33) described groups of sporangia-forming isolates identical to *P. ultimum* in all
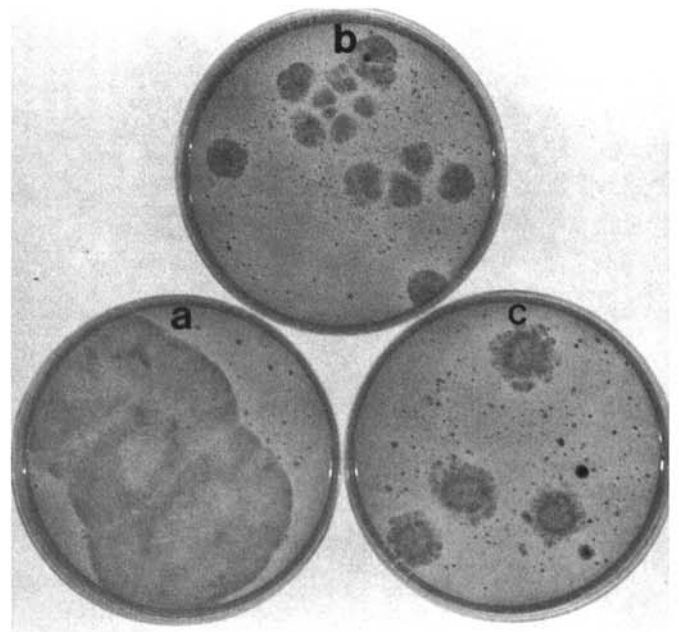

Fig. 1. Colonies of *Pythium* sp.: **a**, isolate N1; **b**, isolate N2; and **c**, isolate N3 after 4 days of incubation on selective medium at 26 C.

characteristics except that oogonia were not produced. Campbell and Sleeth (5) also found such isolates and were able to induce oospore-forming colonies of *P. ultimum* to form only asexual fruiting bodies identical to those from colonies isolated from field soil. A large-colony type (>50 mm after 4 days of incubation at 26 C) similar to N1 (Fig. 1a) was also isolated from the same soils by direct plating on a medium (31) selective for *Pythium* spp.

Two isolates of another *Pythium* sp. also were recovered from soil A and were designated isolates N2 and N3. In contrast to N1, these formed small colonies <25 mm in diameter after 4 days of incubation at 26 C (Fig. 1b and c). Both isolates had a rosette habit of growth on potato-dextrose agar. They grew at temperatures ranging from 7–40 C with optimal radial growth at 30 C. Sporulation did not occur on oatmal agar or V8 broth but was abundant on water-culture with sterile bean-leaf fragments after 14 days of incubation at 26 C in darkness. "Hyphal-swelling," or sporangia, were formed more frequently in isolate N2 than in N3. Sporangia were acrogenous and spherical or intercalary and lemon-shaped, measuring 12–18.5 μm (average 13.2 μm). Sporangia germinated by germ tube and no zoospore production was observed. Oogonia, which formed more frequently in isolate

N3, were acrogenous, smooth, and spherical measuring 18.5–23 μm (average 20.8 μm). Generally one, but occasionally two to five, antheridia per oogonium were observed. Oospores were aplerotic, measuring 18–20 μm (averaging 19.2 μm). The morphology and physiological behavior of these isolates did not match descriptions of any reported species of *Pythium*.

Effect of incubation temperature, soil pH, and *Trichoderma* spp. on the saprophytic development of *Pythium* spp. in soil. When soil was either amended or unamended with *Trichoderma* spp. and bean leaf meal was added at weekly intervals, there was no effect on the population density of isolate N1 that could be attributed to the addition of these antagonists, even though their population densities in soil ranged from 10^4 to 5×10^5 cfu/g of soil (Fig. 2). In soil to which *Trichoderma* spp. had not been added, population densities of the indigenous *Trichoderma* spp. gradually increased from 10^2 to 2.8×10^3 cfu/g of soil over the 6-wk period.

There was, however, a pronounced effect of pH, temperature, and addition of bean leaf meal on population density of N1 independent of additions of *Trichoderma* spp. Therefore, data from all treatments involving *Trichoderma* spp. were composited and the effects of temperature, soil pH, and the addition of bean leaf meal on population density of isolate N1 were plotted in Fig. 3. After the first week, propagule density of isolate N1 increased from barely detectable levels to 2.3×10^2 cfu/g of soil (pH 7.3) incubated at both temperatures (19 and 26 C). However, a greater increase (5 $\times 10^3$ propagules per gram of soil) was recorded in the acidified soil (pH 5.0) at 26 C. In this treatment a further increase occurred up to the third week (2×10^4 propagules per gram) and then declined to 6
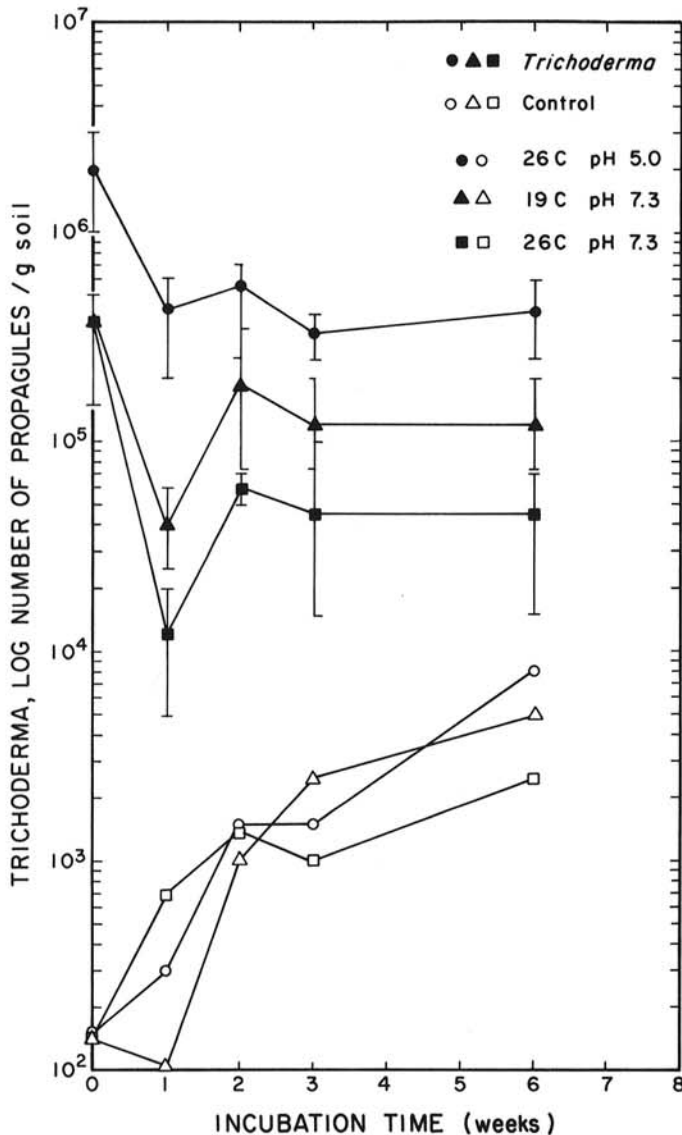
Fig. 2. Effect of incubation temperature and soil pH on population density of *Trichoderma* spp. in soil amended with *Trichoderma harzianum* isolates T12, T95, or *T. koningii* T8 during six weekly repeated incorporations of dried bean leaf meal. Soils were infested with 10^6 conidia of each isolate of *Trichoderma* per gram of soil at the beginning of the experiment. Bars indicate the range of values of colony-forming units per gram of soil in the three treatments (not significantly different).
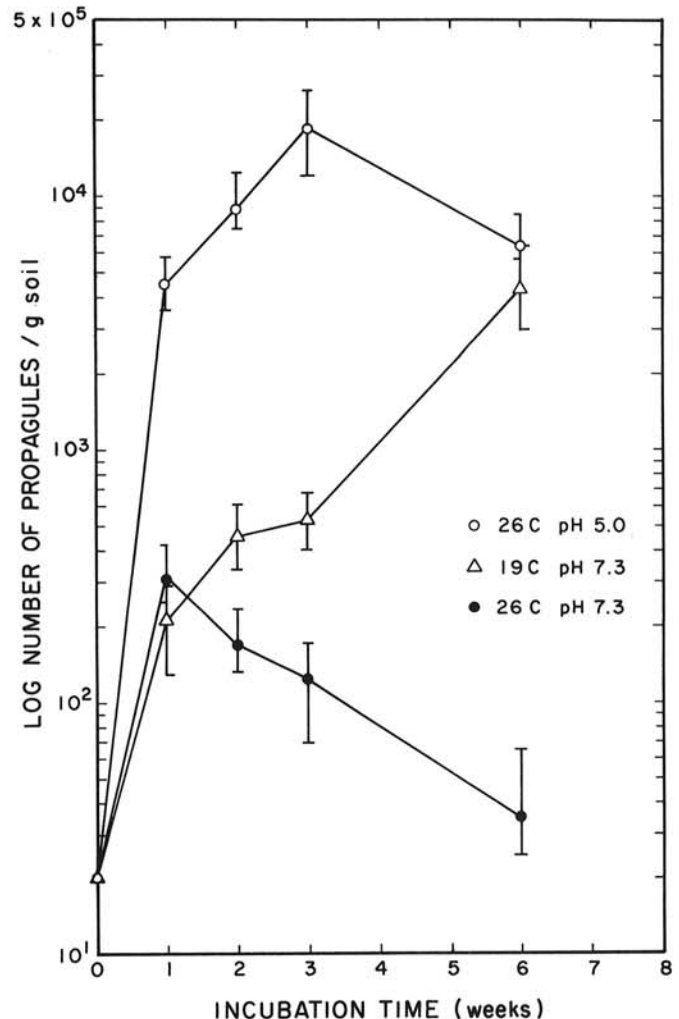
Fig. 3. Effect of incubation temperature and soil pH on propagule density of a pathogenic species of *Pythium* (isolate N1) during six weekly incorporations of dried bean leaf meal (0.3%, w/w). Bars indicate propagule density range (not significantly different) of isolate N1.

$\times 10^3$ propagules per gram after 6 wk. In soil at pH 7.3, N1 propagule density over time was affected drastically by incubation temperature; whereas, at 19 C, population density increased gradually up to 4.5×10^3 propagules per gram after 6 wk. A gradual but steady decline occurred in soil incubated at 26 C. After 6 wk the propagule densities were only five propagules per gram.

At the end of the 6 wk of incubation, counts on a medium selective for *Pythium* sp. were made separately for isolate N1 (large colonies) and for N2 and N3 type (small colonies). At 26 C in soil of pH 5.0 (Fig. 4), there was a high inoculum density of isolate N1 (8.2×10^3 cfu/g soil), whereas population densities of the N2 and N3 types were low (~ 100 cfu/g). However, a reverse situation occurred in soil at the same temperature but at pH 7.3. Population densities of types N2 and N3 of *Pythium* sp. increased to 4.25×10^3 cfu/g of soil, whereas the inoculum densities of N1 declined to less than 50 cfu/g of soil. At the same soil pH (7.3) at 19 C, population densities of N2 and N3 were low, whereas N1 increased to 2.4×10^3 cfu/g of soil.

In vitro interaction between isolate N1 and isolates N2 and N3 of *Pythium* sp. There was no evidence of antibiosis among the different isolates. Under light microscopy, the hyphal side branches of isolates N2 and N3 were finer than those of N1. Therefore, it was possible to distinguish between them in regions of contact. In those regions mycoparasitic activity of isolates N2 and N3 on N1 were apparent. Hyphal interactions occurred from early contact until advanced lysis of the host hyphae (Fig. 5).

Pathogenicity tests. Isolate N1 induced an 80–100% reduction in stand (as compared with uninoculated controls) of the following plants: *Pisum sativum* L. 'Laxton Progress,' *P. sativum* 'Early Frosty,' *P. vulgaris* 'Contender,' *Cucumis sativus* L. 'Straight Eight,' *Citrullus lanatus* L. 'Dixie Queen,' *Cucurbita* sp. 'Bush Table,' *Raphanus sativus* L. 'Early Scarlet Globe,' *Lactuca sativa* L. 'Early Curled,' and *Medicago sativa* L. 'Titan.' Isolate N1 did not significantly ($P = 0.05$) reduce the stands of *Triticum aestivum* L. 'Hermosillo' or *Hordeum vulgare* L. 'Steptoe.' Isolates N2 and N3 of *Pythium* sp. did not significantly ($P = 0.05$) reduce the stands of any of the above plants and there was no evidence of infection by these isolates in any of the potential hosts tested.

Influence of isolates N2 and N3 of *Pythium* on the saprophytic activity of isolate N1. Isolates N2 or N3 were added to soil and incubated for 2 wk, with amendments of bean leaf meal at 0 and 7 days after the beginning of the experiment. In the control soil to which N2 and N3 were not added, inoculum densities of N1 increased from nondetectable levels (<8) to 5.2×10^3 (at 19 C) or 5.0×10^2 cfu/g of soil (at 26 C) during the first week in soil A and to

1.6×10^4 (at 19 C) or 5×10^3 cfu/g of soil (at 26 C) in soil B (Table 1). When isolates N2 and N3 of *Pythium* were added to soil (~ 100 cfu/g of soil), increase in inoculum density of N1 after 1 wk was significantly lower than in soil that had not been infested with these isolates in both soils and at both temperatures. A simultaneous increase (at least to 10^4 cfu/g of soil) was observed in population
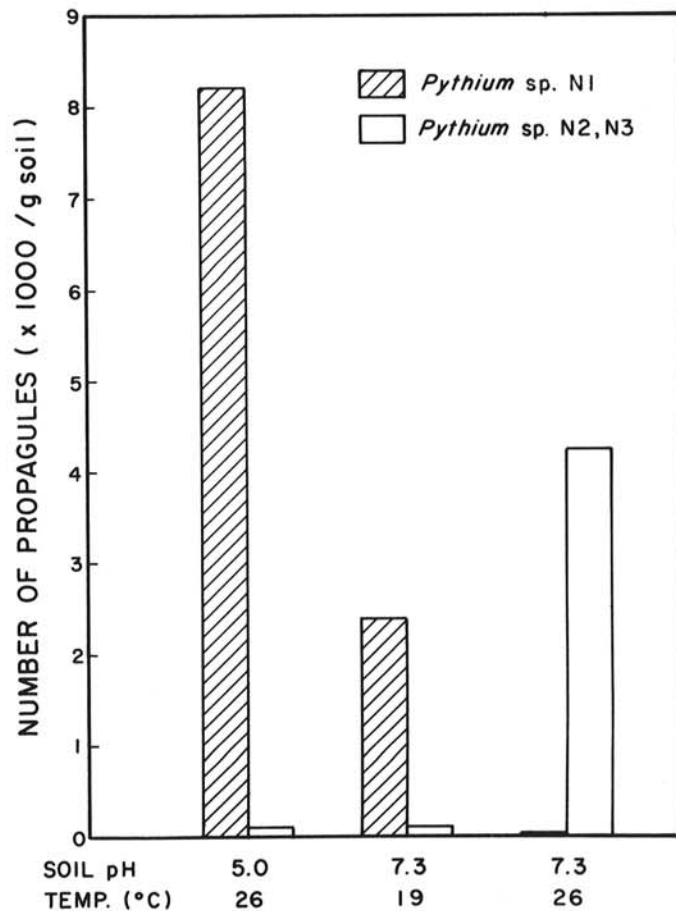

Fig. 4. Effect of incubation temperature and soil pH on *Pythium* spp. propagule densities, as detemined by soil dilutions on a selective medium after six weekly repeated incorporations of dried bean leaf meal to the soil.

TABLE 1. Population densities of mycopathogenic isolate N1 and host isolates N2 or N3 of *Pythium* spp. in soil incubated with two applications of dried bean leaf meal at 0 and 7 days over a 2-wk period

Soil batch[a]	Temperature (C)	*Pythium* sp. isolate added[b]	Incubation time			
			1 wk		2 wk	
			Small colonies[c] (cfu/g × 100)	Large colonies[d] (cfu/g × 100)	Small colonies[c] (cfu/g × 100)	Large colonies[b] (cfu/g × 100)
A	19	None	ND[e] z	52 w	4 y	46 w
		N2	100 v	12 x	380 st	ND z
		N3	160 u	10 x	360 st	ND z
	26	None	0.4 z	5 y	43 w	9 xy
		N2	160 u	0.2 y	460 s	ND z
		N3	190 u	1 z	430 s	0.1 z
B	19	None	ND z	160 u	ND z	150 u
		N2	110 v	4 v	510 s	ND z
		N3	110 v	8 xy	450 s	2.4 yz
	26	None	ND z	50 w	ND z	60 w
		N2	130 v	4 y	430 s	0.7 z
		N3	110 v	2 yz	310 t	ND z

[a] A = Nunn sandy loam collected April 1983; B = Nunn soil collected in July 1982.
[b] *Pythium* isolates N2 and N3 were added to soil so that the population densities were ~ 100 cfu/g soil.
[c] Small colonies <30 mm diameter after 4 days on the *Pythium* selective medium—characteristic of the colony morphology of N2 and N3.
[d] Large colonies >50 mm diameter after 4 days on the *Pythium* selective medium—characteristic of isolate N1.
[e] ND = not detectable; numbers followed by the same letters are not significantly different ($P = 0.05$) according to Fisher's least significant difference test.

densities of isolates N2 and N3. After 2 wk of incubation, a further decrease (in some cases to nondetectable levels) in the inoculum density of N1 in soils infested with isolates N2 and N3 (which again increased their population densities) was detected.

Influence of isolates N2 and N3 of *Pythium* sp. on the incidence of damping-off of cucumber. Isolates N2 and N3 were added to soils A and B and bean leaf meal was added at 0 and 7 days during the 2 wk of incubation. In the controls, isolates N2 and N3 were not added. After incubation, these soils were added to raw soil (soil A) at concentrations of either 1 or 10% by weight. Emergence of cucumber seedlings was consistently and significantly ($P = 0.05$) improved at the 90% dilution over controls to which N2 and N3 were not added (Table 2). Emergence, significantly better than the control, was not consistent at the 90% dilution. Decreasing the percentage of raw soil from 99 to 90% in the mixture unamended with N2 or N3 decreased the percentage of emergence.

Effect of isolates N2 and N3 on lysis of, and the formation of new sporangia by, isolate N1 on membranes placed on soil. Sporangia of isolate N1 placed on nuclepore membranes on soil germinated well (98%) in all treatments (Table 3). However, hyphal lysis was much more advanced after 24 hr on the soil that had been repeatedly amended with bean leaves, whereas a considerable proportion of the hyphae in the nontreated soil remained intact. In this treatment, total lysis occurred at a later stage (48–72 hr). In either case, germinated sporangia were empty, whereas if new sporangia formed on germination hyphae, they were full of protoplasm. A greater number of typical new sporangia were formed per germinated sporangium on the control soil (2.3:1) than on the treated soil (0.38:1) (Table 3 and Fig. 6). On the other hand,

more aborted small sporangia were formed in the amended soil (1.4:1) than in the control soil (0.4:1).

Isolates N2 and N3 of *Pythium* sp. grown in previously autoclaved soil induced germ tube lysis of isolate N1 on membranes resembling that induced on the repeatedly amended soil. They also reduced the number of typical new sporangia formed on the membranes, and increased the number of small ones. Isolate N2 was more effective than isolate N3 in inhibiting the formation of new sporangia. The addition of isolate N1 to the soil (10^4 sporangia per gram of soil) did not induce any change in new sporangia formation.

DISCUSSION

The present study indicated that the presence of *Trichoderma* spp. in relatively high numbers in soil had no effect on the saprophytic development of the naturally occurring pathogenic *Pythium* sp. in contrast to the interaction previously reported between *Trichoderma* spp. and *R. solani* (6,15,16,24). Also, the population densities of the introduced *Trichoderma* spp. did not increase during repeated incorporation of leaf meal (Fig. 2). This may indicate that the introduced isolates did not benefit from the presence of actively growing *Pythium* spp. in the soil as with *R. solani* (16,24). Thus, they cannot be assumed to act as effective biocontrol agents under these soil conditions. There was a slight increase in population densities of native *Trichoderma* spp. in the soil but this was only to relatively low levels, perhaps due to saprophytic utilization of the leaf meal added to the soil.

Soil pH and incubation temperature strongly affected the population density of *Pythium* spp. during saprophytic growth. Propagule density of isolate N1 reached its highest level at a soil pH of 5.0 at 26 C (Fig. 3). The lowest propagule density was at soil pH of 7.3 at 26 C. In vitro studies (J. S. Ahman, *unpublished*) have shown that linear growth rate and sporangial germination of isolate N1 were higher at pH 7.3 than at pH 5.0; thus, the enhanced saprophytic propagule buildup of isolate N1 in raw soil at the acid pH suggests that this soil reaction was acting indirectly by inhibiting antagonistic microorganisms in soil that limits the development of isolates N1 at an alkaline pH and higher temperature. Similarly, soil conduciveness to isolate N1 at the cool temperature (19 C) probably is due to the decreased activity of the antagonistic microflora which is more effective at elevated temperatures (23).

When soil was incubated at 26 C, the population density of isolate N1 was increased initially. However, it decreased later, although a fresh nutrient source was resupplied weekly. Conversely, it coincided with a sharp increase in population densities of the mycoparasitic isolates N2 and N3 of *Pythium* sp. The inverse relationship in population densities between isolate N1 and the mycoparasites (N2 and N3) suggested that the saprophytic development of N1 was directly suppressed by isolate types N2 and N3 of *Pythium* sp. This assumption was supported when preparations of isolates N2 and N3 suppressed the saprophytic development of isolate N1 in raw soil (Table 1).

TABLE 2. Preemergence damping-off of cucumbers induced by *Pythium* sp. (isolate N1) in soil to which isolates N2 or N3 had or had not been added

Soil batch[a]	Raw soil in mixture[b] (%)	Temperature of incubation (C)	Control (%)	N2 (%)	N3 (%)
			Healthy plants		
A	90	19	46.7 y	86.7 v	90.0 v
	99	19	70.0 wx	83.3 v	90.0 v
	90	26	60.0 x	90.0 v	86.7 v
	99	26	83.3 v	83.3 v	90.0 v
B	90	19	30.0 z	90.0 v	70.0 wx
	99	19	66.7 x	90.0 v	63.3 x
	90	26	36.7 z	76.7 v	76.7 w
	99	26	63.3 x	90.0 v	83.3 v
A (nontreated)			90.0 v		

[a] A = Nunn soil collected April 1983; B = Nunn soil collected in July 1982.
[b] Isolates N2 and N3 were added to raw soils A and B and leaf meal was added at two weekly intervals during the 2 wk of incubation. In controls, N2 and N3 were not added. These soils were added to raw soil (A) at concentrations of either 1 or 10% by weight.
[c] Numbers followed by the same letters are not significantly different ($P = 0.05$) by use of Fisher's least significant difference.

TABLE 3. Effect of six weekly incorporations of bean leaves (0.3%, w/w) to soil on germination and new sporangia formation by sporangia of *Pythium* sp. (isolate N1) placed on membranes on soil

			Number sporangia/membrane[b]				
			Typical			Small	
	Germination (%)				Ratio		Ratio
Soil treatment[a]	24 hr	Empty	New 20-24 μm	New:Empty		New 10-15 μm	New:Empty
Nonamended	98	675 z[d]	1,564 z	2.3:1 z		245 y	0.4:1 x
Amended with bean leaves only	98	603 z	230 y	0.4:1 y		842 y	1.4:1 y
Pythium sp. N1[c]	98	775 z	1,742 z	2.5:1 z		302 x	0.4:1 x
Pythium sp. N3	98	627 z	132 x	0.4:1 y		1,110 z	1.8:1 z
Pythium sp. N2	98	639 z	49 w	0.1:1 x		837 y	1.3:1 y

[a] Bean leaves (0.3%) were added to all treatments before placing the Nuclepore membranes on soil (batch A).
[b] All sporangia were counted on each membrane after 48 hr of incubation. Empty sporangia were the germinated ones and the new were the newly formed ones. Two membranes were counted for each treatment. The experiment was repeated three times.
[c] Sporangia of isolate N1 added to raw soil (10^4 sporangia/g soil).
[d] Numbers in each column followed by the same letters are not significantly different ($P = 0.05$) by use of FLSD.

Proof of causality and the elaboration of mechanisms involved in the induction of suppressiveness are difficult to identify concisely due to the multiple factors involved (2,24). The mechanism of suppression in this system could involve competition (1) since taxonomically and/or ecologically related microorganisms may occupy similar ecological niches. There are also two indications that mycoparasitism could be involved; microscopic observations revealed N2 and N3 of *Pythium* sp. parasitizing hyphae of isolate N1 (Fig. 5), and, following an initial increase, the propagule density of N1 actually decreased in the presence of N2 and N3 (Fig. 3) in spite of repeated incorporation of bean leaves (Table 1).

Hyperparasitism may not be the only antagonistic mechanism involved in suppression. Lysis of sporangial germ tubes of N1 occurred earlier when sporangia on membranes were placed on a repeatedly amended soil for 6 wk than on raw soil (Table 3). Similar lysis occurred in autoclaved soil infested with types N2 and N3 of *Pythium* sp. but N3 was less effective. Sporangia on membranes placed on soil previously amended for 6 wk with bean leaf meal formed fewer new typical sporangia than on raw soil. Moreover, more aborted new sporangia were observed on the former treatment and on sterile soil infested with isolate N2 and N3. Sporangia of isolate N1 added to soil did not affect new
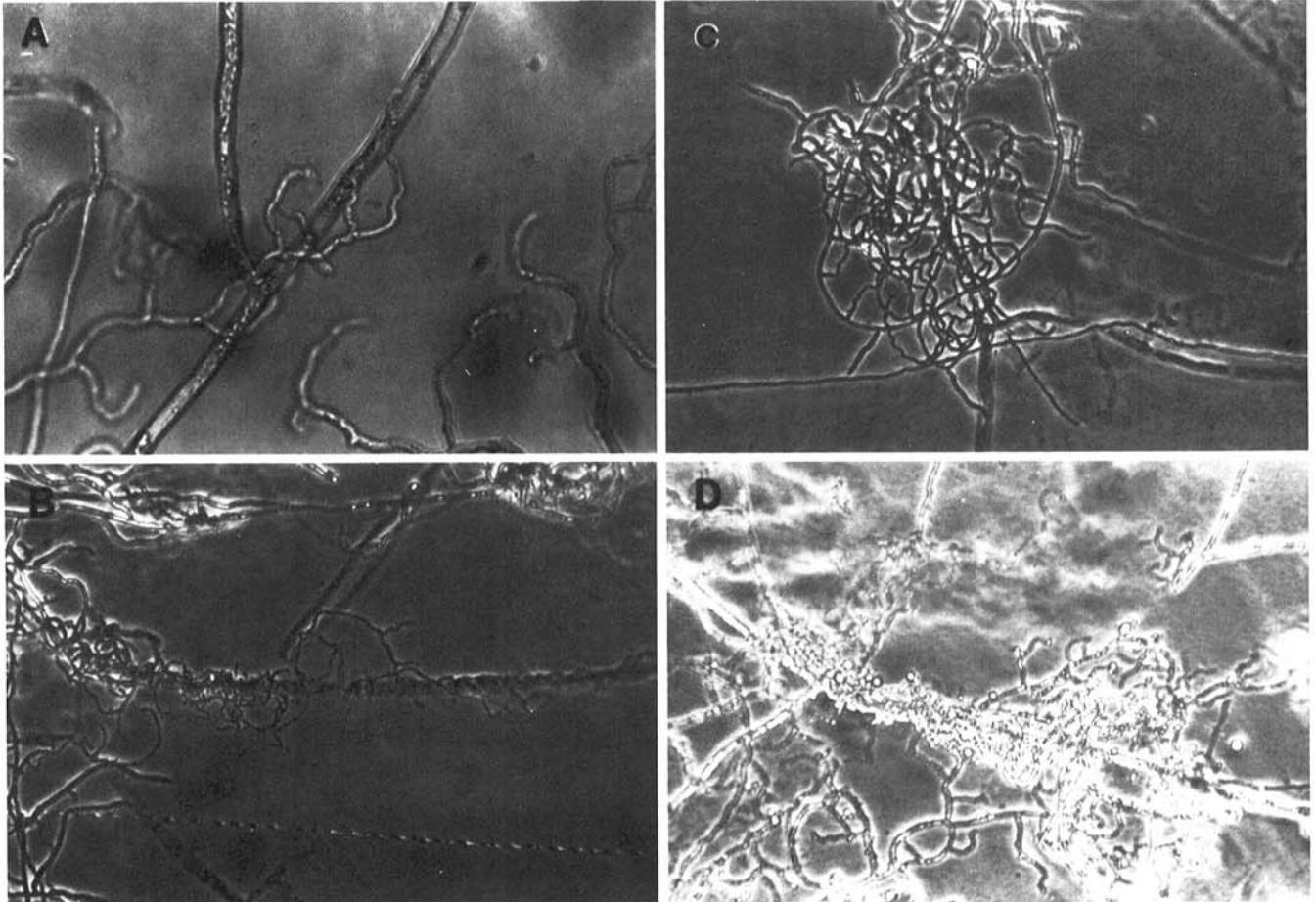

Fig. 5. Interaction between hyphae of the mycoparasite (isolates N2 and N3 of *Pythium* spp.) and host (isolate N1 of a *Pythium* sp.) at an early stage (**A and B**) and at advanced stages of development. (**C and D**), A massive growth of the mycoparasites is associated with lysis of the host hyphae.
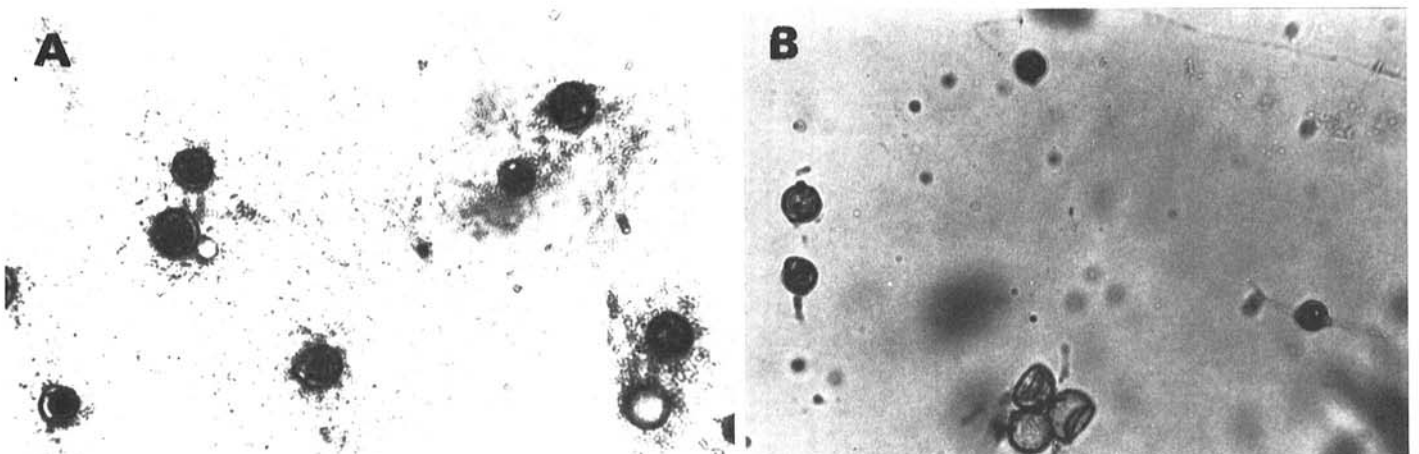

Fig. 6. Germinated (empty) and secondary sporangia of isolate N1 of *Pythium* sp. on Nuclepore membranes incubated for 24 hr on soil; **A,** soil not previously amended; **B,** soil previously amended with six incorporations of dried bean leaf meal at 1-wk intervals. Sporangia formed from germ tubes are stained. In **B** they are fewer in number and smaller than in **A.**

sporangium formation on membranes indicating that simple competition for nutrients was not involved. However, the effect of N2 and N3 apparently was exerted through the membranes, suggesting that a diffusible substance (enzymatic or toxic) is also involved in the destruction of the mycelium of isolate N1 after germination.

The reduction in inoculum density of isolate N1 of *Pythium* sp. in the presence of isolates N2 and N3 was paralleled by a reduction in incidence of cucumber preemergence damping-off (Table 2). However, when isolates N2 and N3 were not added to soil amended over 2 wk with bean meal, addition of this mixture to raw soil at a level of 10% resulted in higher incidence of disease than at 1%. This suggests that isolate N1 increased during the 2-wk incubation period to levels (Fig. 3) sufficient to induce significant disease even when the infested soil was diluted with raw soil.

Pathogenicity tests of isolates N1, N2, and N3 of *Pythium* indicated that while N1 was pathogenic to the plants tested except wheat and barley, the other two isolates were not. These findings suggest the potential use of isolates N2 and N3 as biocontrol agents against pathogenic *Pythium* sp. The feasibility of biological control was supported further by the capacity of isolates N2 and N3 to induce suppressiveness in soil to damping-off of cucumber (Table 2). Unpublished results also indicated that isolates N2 and N3 were also mycoparasitic to several other *Pythium* spp., *Phytophthora* spp., and *R. solani*.

Bouhot and Perrin (4) described the occurrence of soils in which inoculum potential of pathogenic *Pythium* spp. decreased in time. Similarly, Watson (40) showed that inoculum potential of *P. ultimum* was initially increased but then decreased in time, following the introduction of plant residues into the soil. In both cases it was suggested that soil suppressiveness was induced by a biological mechanism, but the responsible agents were not identified. Recently Kao and Ko (19) described a naturally occurring soil suppressive to damping-off of cucumber induced by *P. splendens*. It was suggested that a combination of microbiological and abiotic factors were responsible for the suppressiveness. While suppressiveness in all these examples was thought to be induced, at least partially by the biological elements of the system, the causal agent was not identified.

Pythium spp. described as potential antagonists to plant pathogens are *P. oligandrum*, *P. acanthicum*, and *P. periplocum*; all of these are characterized by spiny oogonia (8–10,18,39). Whereas most of the reports described only in vitro hyphal interactions, Vesely (38) reported the potential use of *P. oligandrum* in biological control. He observed that the fungus could reduce the damage caused in sugar beet seedlings by *P. debaryanum* and *P. ultimum* while causing little or no damage itself. Martin and Hancock (28–30) found that *P. oligandrum* was among the principle competitors to *P. ultimum* in the San Joaquin Valley in California. They suggested that *P. oligandrum*, favored by high concentrations of soluble chloride ions in soil, suppressed the saprophytic growth of *P. ultimum*. Sneh et al (31) demonstrated parasitism of oospores of a *Pythium* sp. by another *Pythium* sp. and suggested the interaction of a variety of mycoparasites with *Phytophthora* spp., *Pythium* spp., and *Aphanomyces euteiches*; however, isolates N2 and N3 do not resemble any of the *Pythium* spp. mentioned above.

The primary focus of the research reported here was to discover the mechanisms associated with the described suppressive soil system. These basic findings, however, point to certain potential advantages of using isolates N2 or N3 in agricultural applications: as mycoparasites they may reduce the density of an established inoculum of pathogenic *Pythium* species in soil; they increase their population density in raw soil amended with bean leaf meal, suggesting a strong competitive saprophytic ability in soil; since they are taxonomically related to and apparently occupy the same substrates in soil as pathogenic *Pythium* spp., they are likely to function as strong competitors and, therefore, to suppress inocula buildup of pathogenic *Pythium* species in soil.

Successful control of damping-off induced by *Pythium* spp. was achieved by seed coating with a variety of antagonistic microorganisms (14,19,20,25,26,32,39). However, seed treatment generally provides a temporary protection for only a short period of time and requires repeated applications for every crop. Conversely, control of *Pythium* spp., by direct application of antagonists to raw soil (17) requires relatively large amounts of a biocontrol agent. The present study suggests a procedure to induce suppressiveness by an intial application of a relatively small density of biocontrol agent followed by organic amendment to provide a propagule buildup of the biocontrol agent in the soil. Such a procedure also has a potential for a long-term effect.

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