Disease Progression by Active Mycelial Growth and Biocontrol of *Pythium ultimum* var. *ultimum* Studied Using a Rhizobox System

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ABSTRACT

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This study demonstrates that outward growth of mycelium from primary foci through bulk potting mix to roots of adjoining plants can be an important means of spread of damping-off and root rot caused by *Pythium ultimum*. The use of a rhizobox system, which confines plant roots, enabled us to study the spread of actively growing mycelium between root systems placed at precise distances from each other. In steamed potting mix, hyphae of *P. ultimum* on average grew 9.6 cm from diseased root tissue compared to 5.3 cm in raw potting mix. The density of mycelium was highest within the first 2 cm from the infected root tissue, decreasing with increasing distances from the roots. Accordingly,

the disease on adjacent plants decreased as the distance from infected roots increased. The time required for damping-off of adjacent plants was 3 days slower in raw as compared to steamed potting mix and increased by 2 days for each additional centimeter between the rhizoboxes. The presence of *Trichoderma harzianum* diminished the production of secondary inoculum and reduced the ability of *P. ultimum* hyphae to extend through bulk potting mix. In conclusion, the concentration of the primary inoculum, the plant density, the distance separating diseased from healthy roots, the resident microflora, and the presence of an antagonist were shown to be important factors affecting disease spread by mycelial growth.

Additional keywords: damping-off, oospore, root rot, *Trichoderma harzianum*.

Pythium ultimum is a widespread soilborne pathogen that attacks a large number of plant species (14). Generally, *Pythium* spp. have a preference for young or succulent host tissue commonly infecting seeds and the radicals causing seed rot and pre-emergence damping-off. These fungi also infect newly emerged seedlings at ground level, causing post-emergence damping-off. At later stages, when cells of stems and main roots on host plants have developed secondary wall thickenings, infection is restricted to root tips, or feeder roots limiting plant vigor and yield, and sometimes causing plant death. *Pythium* spp. are frequently found in glasshouses, and horticultural and forest tree nurseries where soil moisture and plant density is high, providing a favorable environment with masses of succulent roots and associated young stem tissue that are vulnerable to attack (15). We have observed that under these conditions the disease begins on one or a few plants and expands to adjacent plants resulting in damping-off of a large number of plants in restricted areas.

Oospores are initially thick-walled, resistant to desiccation, and survive for long periods in dry soil (16). Together with sporangia (24), they are considered the primary survival structures and sources of inoculum of several *Pythium* spp. These structures may overcome fungistasis and germinate directly via a germ tube in response to exogenous carbohydrates and amino acids diffusing from host plants (23) or from volatile seed exudates produced during seed germination (19). Under appropriate environmental conditions, sporangia or oospores of some *Pythium* spp. may germinate indirectly and produce zoospores. While oospores and sporangia have limited mobility, the biflagellate zoospores are very mobile

in water. Once a plant has been infected by primary inoculum, most *Pythium* spp. produce secondary inoculum which spreads from these primary foci to neighboring plants (26). Under appropriate environmental conditions, especially the presence of a high ambient water potential (11), it is thought that zoospores account for most of this secondary inoculum. Where movement of water occurs, zoospores can spread over entire greenhouses (25,26). In contrast, in stagnant water, the distance traveled by the individual zoospore is usually relatively short (<10 cm) despite their motility (6,18). Spread of zoospores through soil and growth media is even more limited and depends on the water potential and the texture of the soil and growth medium (9). Species of *Pythium* such as *P. ultimum* var. *ultimum* rarely produce zoospores (28), but apparently are capable of spreading from primary foci to adjacent plants.

Based on these arguments, it is likely that active growth of mycelia from infected plant tissue may be important as a means of progression of damping-off and root rot especially under conditions such as low water potentials and fine texture growth media restricting the discharge and spread of zoospores. However, very few papers have addressed this subject and to our knowledge none have dealt with fungi from the genus *Pythium*. Consequently, the significance and the nature of such spread are poorly understood.

The present work was carried out to test the hypothesis that spread of active growing mycelium through potting mix is an important means of spread of *P. ultimum*. The primary objective was to investigate the possibility and nature of such spread, including the effects of the concentration of the primary inoculum, the plant density, the distance between roots, and the resident microflora. The secondary objective was to study the ability of *Trichoderma harzianum* to suppress production and spread of mycelial inoculum. A rhizobox system, which confines plant roots thereby enabling root systems to be placed at precise distances from each other is described for the first time.

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MATERIALS AND METHODS

Fungi. *Pythium ultimum* Trow var. *ultimum* isolate HB2 originally was isolated from a sandy loam soil from Højbakkegård, Tåstrup, Denmark (29). The fungus was stored on autoclaved barley leaves kept in sterile tap water at 4°C until use.

Trichoderma harzianum Rifai isolate T3 originally was isolated from a *Pythium*-suppressive peat (29). Peat-bran inoculum of T3 was prepared according to Sivan et al. (22).

Plants. Cucumber plants, *Cucumis sativus* L. cv. Langelands Kæmpe - Gigant (Dæhnfeldt, Odense, Denmark) were used for all experiments. Before use, cucumber seeds were surface-disinfected in 2.5% NaOCl (Bie and Berntsen, Rødovre, Denmark) and pregerminated at 25°C for 3 or 4 days on half-strength potato dextrose agar, (PDA; 18.75 g of PDA, 12.5 g Bacto [Difco Laboratories, Detroit, MI] agar, 1 liter of H₂O). Seedlings with a uniform size were selected.

Oospore inoculum. Potting mix (see below) was powdered in a coffee mill (Krups, type 203B, France) for 30 s and the equivalent of 1.5 g of dry matter and 10 ml of deionized water were placed in 250-ml Erlenmeyer flasks and autoclaved for 20 min. The following day, 40 ml of potato carrot broth, (PCB; 21) was added and the flasks were autoclaved again for 20 min. The pH of the substrate was 5.5. The substrate was then inoculated with one small agar block (1 mm³) cut from the edge of an actively growing colony of *P. ultimum* HB2. The flasks were incubated in the dark at 20°C for 7 days. At harvest, the contents of the flasks were poured into a sterile 10-cm Büchner funnel with a cellulose filter (1-F-1445, Bie and Berntsen), and washed with sterile, deionized water to remove excess PCB. The inoculum was dried in a laminar airflow bench (Holten LaminAir A/S, Allerød, Denmark) for 2 days, crushed in a sterile mortar, and stored in a sterile glass container in darkness at 20 to 25°C for less than a month. The inoculum was checked for bacterial contamination by culturing in 2 ml of Bacto (Difco) nutrient broth (8 g per liter). The oospore concentration was assessed by grinding the dried inoculum thoroughly in a mortar with successive additions of water to reach a suspension with a final concentration of 0.0035 g per ml. The suspension was placed on a hemacytometer and the number of thick-walled oospores was determined. Viability was tested by phloxine B staining (20). Inoculum for control treatments was harvested after 2 days of incubation before formation of oogonia had commenced.

Fig. 1. Illustration of a rhizobox. The box was made of a nylon mesh, with a pore size of 38 μ m, which was stretched over a plastic frame. The resulting open-topped chamber measured 1 $\times$ 7 $\times$ 7 cm. The mesh confined the roots while nutrient solutions could move and microorganisms could grow through the mesh.

Viability of *P. ultimum* HB2 in inoculum for control treatments and germinability of oospores were observed microscopically after mixing the inocula with steamed potting mix watered with sterile cucumber seedling extract and incubated at 18°C for 3 days. The seedling extract was prepared by soaking approximately 75 4-day-old cucumber seedlings in 400 ml of sterile dilute salts (DS) (10) for 2 days at 4°C. The extract was sterilized by filtration through a 0.45 μ m Minisart, NML, cellulose acetate filter (Sartorius, Göttingen, Germany).

Potting mix and growth conditions. Unless otherwise stated, plants were grown in 500-ml sterile pots containing a total of 30 g of dry matter of a fine, 0- to 20-mm, light-colored sphagnum peat, Færdigblanding 1, (Pinstrup, Mosebrug A/S, Ryomgaard, Denmark) mixed with vermiculite, grade 2 (Skamol, Nykøbing Mors, Denmark), at 5:2 (wt/wt). The final pH (H₂O) was 5.5. Both steamed and raw potting mix were used. Steaming was conducted in autoclave bags (Scienceware, Pequannock, NJ) at 100°C for 1 h on three successive days. After planting, the pots were watered with a sterile balanced nutrient solution containing (mM): Ca(NO₃)₂ (2.2); NH₄NO₃ (1.3); KNO₃ (1.1); Mg(NO₃)₂ (1.0); KH₂PO₄ (0.7); MgSO₄ (0.38); Fe³⁺-EDTA (0.0016); MnSO₄ (0.0023); H₃BO₃ (0.0065); ZnSO₄ (0.0024); NaMoO₄ (0.0003); NaCl (0.01); CuSO₄ (0.0007) to a water content of -0.98 kPa (63.6% volume) and a conductivity of 3.5 mMho. The pots were placed in a growth chamber at 18°C with a 16 h photoperiod and, unless otherwise stated, were watered to the initial weight on alternate days.

The rhizobox and inoculation of plants. To prevent disease spread through root contact, plants in most experiments were grown in rhizoboxes (Fig. 1) made of a 1-cm-wide plastic frame, which was inserted into a fine mesh (38 μ m) nylon bag. The resulting open-topped chamber measured 1 $\times$ 7 $\times$ 7 cm. The fine mesh confined plant roots, while fungal mycelia could grow through the mesh and the nutrient solution move freely between the rhizobox and the surrounding potting mix. Eight 3-day-old seedlings were planted into each rhizobox containing potting mix (3.75 g of dry matter content). Inoculated plants were grown in rhizoboxes containing potting mix thoroughly mixed with oospore inoculum to a concentration of 5 $\times$ 10⁴ oospores per g unless otherwise stated. The high oospore concentration was used to ensure damping-off. After 5 days, when all inoculated plants were severely diseased, the rhizoboxes with plants were transferred to new pots and set-up with the actual treatments of the experiments (see below). In order to study the spread from diseased roots to healthy roots of adjacent plants, rhizoboxes with inoculated plants were placed at precise distance from rhizoboxes containing noninoculated plants by use of plastic-spacers (Fig. 2).

Plant analysis. Harvested plants were carefully rinsed in water and the severity of disease on roots and collar was rated according to the following scale modified from Wolffechele (29): 0 = healthy looking, heavily branched root system; 1 = root system less branched than healthy roots (rating 0), light brown necrosis in distinct spots, often associated with the tip of side roots; 2 = only few side roots, and dark brown necrosis in distinct spots; 3 = few and small side roots, and dark brown necrosis of the entire root system, or all around the stem; 4 = plant dead.

The mean disease index of the plants in each rhizobox or pot was calculated, representing one replicate.

Population density of *P. ultimum*. Population densities of *P. ultimum* HB2 were assessed by sampling 10 g of either bulk potting mix or potting mix from the root zone; the latter by separating the rhizoboxes and carefully removing the potting mix from the roots. Samples were then suspended in 100 ml of DS and homogenized in an Ultra-turrax T 25 (IKA-Labortechnik, Staufen, Germany) for 2 min at 13,500 rpm. Serial dilutions were made and 100- μ l aliquots were plated on *Pythium* selective medium, P₁₀VP, (0.2 g of 50% brassicol, 0.01 g of rose bengal [Sigma Chemical Company, St. Louis], 0.01 g of 85% pimarizin [Merck, Darmstadt, Germany], 0.2 g of vancomycin [Eli Lilly and Co., Indianapolis],

17 g of corn meal agar [Difco], and 1 liter of demineralized water [27]). The populations were expressed as colony forming units (CFU) per gram of dry potting mix. The dry weight was determined from a duplicate 10-g sample.

Interaction between seedling density and inoculum concentration. This experiment was carried out to investigate the influence of plant density and concentration of the primary inoculum on progression of damping-off and root rot. The experiment had a complete factorial design with nine main treatments (three oospore concentrations $\times$ three plant densities) and each main treatment had four replicates (i.e., a total of 36 pots). The experiment was carried out in steamed potting mix and repeated three times. Preliminary experiments showed that due to variable germination of the oospores, high inoculum levels were necessary in order to obtain a consistent level of disease development. The oospore concentration was 0, 9,000, and 18,000 oospores per g in two of the repeated experiments (Fig. 3A), and 0, 22,000, and 30,800 oospores per g in the third repetition (Fig. 3B). For each inoculum concentration, 4, 8, or 16 4-day-old seedlings were planted in the pots. After 14 days, disease severity on roots and collar was rated as described above.

Spread of *P. ultimum* in bulk potting mix. The purpose of this experiment was to determine if *P. ultimum* spreads from infected root tissue by active mycelial growth. The maximal distance of the spread and the densities of *P. ultimum* HB2 at different distances from the roots were assessed. Additionally, the influence of *T. harzianum* T3 and the resident microflora on both spread and density of *P. ultimum* was evaluated. The experiment had a complete factorial design with eight main treatments ($\pm$ steaming $\times \pm$ T3 $\times \pm$ HB2). Each main treatment had three replicates (i.e., a total of 24 pots). The experiment was repeated three times. Rhizoboxes with severely diseased plants (disease index between 3 and 4) were transferred, 5 days after the seedlings had been planted, to one end of 1 liter, 15-cm-long, sterile plastic containers which were filled with additional potting mix (83 g of dry matter content), either noninfested or infested to a final concentration of 3×10^8 CFU per g of dry matter using a peat-bran preparation of *T. harzianum* T3. Water was added to the containers in the distant end of the one with the rhizobox and only once when setting-up the experiment. All containers were covered with sterile plastic

Fig. 2. Experimental set-up used to study the spread of *Pythium ultimum* (HB2) by active mycelial growth from diseased roots to roots of non-inoculated plants. Rhizoboxes with plants could, by use of plastic-spacers, be placed at a precise distance (here 2 cm) from each other.

bags during incubation. After 14 days, samples of potting mix were taken at different distances from the rhizoboxes, and placed on P₁₀VP. Three samples were taken per location. After 2 days of incubation, it was determined whether *P. ultimum* HB2 grew on the plates (qualitative determination). Indigenous *Pythium* spp. were distinguished from HB2 on the basis of growth morphology and pathogenicity. In one of the experiments, the population density of *P. ultimum* was determined by dilution plating as described above (quantitative determination).

In order to study the morphological development of *P. ultimum* HB2 in bulk potting mix, microscopic examinations were carried out at days 2, 4, 7, and 14 after transfer of the rhizoboxes. Approximately 2-g samples of the potting mix were taken at different distances from the rhizobox, and ground in a mortar with a pestle under successive addition of deionized H₂O. Ten drops of 1% aniline blue in lactophenol were added and the suspension was allowed to sit for 5 min. After transfer to a glass tube, the suspension was allowed to settle for 30 s before a 0.5-ml sample was taken from the upper layer of the suspension and filtered through a 3- μ m cellulose nitrate filter (Sartorius). The filter was mounted in a drop of H₂O on a glass slide, covered with a cover slip, and viewed under a Nikon Optiphot microscope (Nikon, Tokyo). To rule out the presence of zoospores, samples containing soil water, potting mix, and plant roots were examined with an Olympus SZH stereomicroscope (Olympus, Tokyo) on the above mentioned days.

Root-to-root spread of *P. ultimum*. The purpose of this experiment was to investigate the impact of the distance between diseased and healthy roots on pathogen spread. The influence of the resident microflora also was evaluated. The experiment had a complete factorial design with 12 main treatments (three distances $\times \pm$ sterilization $\times \pm$ HB2). Each main treatment had three replicates (i.e., a total of 36 pots). The experiment was repeated twice. Rhizoboxes with inoculated plants were placed in pots opposite rhizoboxes containing noninoculated plants. Plastic-spacers ensured that distances of 1, 2, or 3 cm were established between the two rhizoboxes (Fig. 2). The length of time (days) required until the first of the noninoculated plants damped-off was recorded, and after 14 days, disease severity on roots and collar was rated as described previously.

Fig. 3. Relationship between concentration of *Pythium ultimum* (HB2) oospores, disease severity, and number of cucumber seedlings (seedl.) per pot. The seedlings were grown in steamed potting mix. This figure is based on data from three repeated experiments. Section A shows the average of two repeated experiments with 0, 9,000, and 18,000 oospores per g of potting mix. Section B is based on the third repetition with 0, 22,000, and 30,800 oospores per g of potting mix. The disease index relates to symptoms on roots and collar. The disease index scale can be found in text. Bars represent the standard error.

Suppression of production and spread of secondary inoculum of *P. ultimum*. The objective of this experiment was to study the ability of *T. harzianum* to reduce production and spread of secondary inoculum of *P. ultimum* from primary foci to healthy roots of adjacent plants. The experiment was repeated twice, although the first repetition had an incomplete design. The second repetition had a complete factorial design with six main treatments. Each main treatment had three replicates (i.e., a total of 18 pots). Only results from the second repetitive experiment, which are representative of both, are presented. Steamed potting mix was used for this experiment and thoroughly mixed with inocula of *P. ultimum* HB2 and *T. harzianum* T3 to concentrations of 3×10^4 oospores or 3.6×10^4 CFU per g of dry matter, respectively. Rhizoboxes with inoculated plants were placed opposite rhizoboxes containing noninoculated plants. Plastic-spacers ensured that a distance of 2 cm was established between the two rhizoboxes (Fig. 2). The following describe the different treatments, i.e., addition of T3 and/or HB2 to box 1, between the boxes, and box 2, respectively: (box 1|between boxes|box 2): -|-|-; T3|T3|T3; HB2|-|-; HB2|T3|T3; HB2+T3|T3|T3 (also described in the corresponding table). For each of these five treatments, five plants were grown in each box. For the sixth treatment (HB2|-|-), no plants were grown in box 1. This last treatment was included as a control in order to test possible diffusion spread of oospores. On the day of transfer (after 5 days) and when the experiment was terminated (after 12 days), potting mix from rhizoboxes was sampled and used for dilution plating on P₁₀VP, as described above. Disease severity on the roots and collars of the harvested plants was rated both at day 5 and day 12.

Statistical analysis. All calculations were carried out using the Statistic Analysis System, version 6.12 (SAS Institute, Cary, NC). For all experiments the levels of significance for the experimental repetitions, main treatments, and their interactions were calculated using the General Linear Models Procedure (PROC GLM). Since the quantitative data, concerning the spread of *P. ultimum* in bulk potting mix, were interdependent, the population densities belonging to each distance were pooled and transformed logarithmically before analysis. For the experiment concerning the interaction between seedling density and inoculum concentration, the test was based on a model assuming that there was a linear effect of inoculum concentration with a slope depending on the seedling densities (Fig. 3). Similarity of the slopes was analyzed using PROC GLM. Correlation between the disease index and the population density of *P. ultimum* in the root zone was tested by regression analysis (PROC REG).

RESULTS

Oospore inoculum. Synchronous formation of oogonia started on the fourth day of incubation of the oospore inoculum at 20°C. At harvest, the concentration of oospores per g of dried inoculum averaged 10^6 , and varied between batches within two orders of

magnitude. Approximately 98% of the oospores were viable and had thick intact walls, demonstrated by their inability to take up phloxine B. The remaining 2% had disintegrated. The control inoculum, which was harvested after 2 days of incubation and before formation of oogonia had commenced, never gave rise to growth on any solid media or when mixed in steamed potting mix and watered with cucumber seedling extract. Nor did the control inoculum cause any disease when added to the test plants. This indicates that drying killed the mycelium. When the oospore inoculum was mixed in the steamed potting mix watered with cucumber seedling extract germination and growth occurred. The germinability was variable and precise determination of percent germination was not possible as many oospores were hidden within peat particles. Based on an approximation, more than 60% of the oospores germinated and all of those germinated directly with a germ tube.

Interaction between seedling density and inoculum concentration. The statistical analysis showed a significant difference ($P = 0.005$) between the experimental repetitions. This significant difference was caused by the use of a higher inoculum concentration in the third experimental repetition (Fig. 3B) than in the two first repetitions (Fig. 3A). Disease severity on cucumber seedlings grown in steamed potting mix increased significantly both with enhanced oospore concentration ($P < 0.001$) and seedling density ($P < 0.001$) (Fig. 3). There was a significant interaction ($P = 0.005$) between oospore concentration and seedling density; the enhancement in disease severity due to increased seedling density was greatest at the lower oospore concentrations. There was no effect of the seedling density at the highest oospore concentration (30,800 spores per g).

Spread of *P. ultimum* in bulk potting mix. Raw potting mix contained indigenous species of *Pythium*. These were, however, easily distinguishable from *P. ultimum* HB2 based on growth morphology. The population densities of the indigenous *Pythium* spp. were less than 100 CFU per g and did not interfere with the population studies of *P. ultimum* HB2, nor did they cause any disease on the test plants. There was no significant difference in the content of the indigenous *Pythium* spp. between the three experimental repetitions.

In steamed potting mix, *P. ultimum* was, on average, isolated 9.6 cm from rhizoboxes containing diseased plants (qualitative determination). However, the distance of spread was significantly less ($P = 0.005$) in raw potting mix where *P. ultimum*, on average, grew 5.3 cm from the diseased roots. There was a significant interaction ($P = 0.027$) between sterilization and *T. harzianum*, because *T. harzianum* reduced the spread of *P. ultimum* by 42% compared to the treatment without *T. harzianum*, but only in steamed potting mix.

Population studies (quantitative determination) showed that the density of *P. ultimum* was greatest within 2 cm from the infected root systems, and decreased with increasing distance from the roots (Table 1). This was the case both in steamed and raw potting mix. The total population of *P. ultimum* in the containers was sig-

TABLE 1. Spread of *Pythium ultimum* (HB2) from diseased roots into bulk potting mix (quantitative determination)^a

Treatment ^b	Population density (CFU per gram of potting mix) of HB2 ^c							LSD _{0.05}
	0-2 cm	2-4 cm	4-6 cm	6-8 cm	8-10 cm	10-12 cm	12-14 cm	
Raw								
- T3	18,820	630	16	0	0	0	0	7,397
+ T3	5,510	579	16	0	0	0	0	883
LSD _{0.05}	18,032	560	42	0	0	0	0	...
Steamed								
- T3	2,798	70	75	29	11	8	0	693
+ T3	4,387	83	0	0	0	0	0	615
LSD _{0.05}	2,238	111	90	66	31	21	0	...

^a Three repetitive experiments were carried out with similar results. The population density as presented above was only determined in one of the repetitions.

^b The potting mix was either steamed or raw, and infested or noninfested with *Trichoderma harzianum* (T3).

^c Spread of HB2 was measured by dilution plating of samples collected at different distances from infected roots of plants grown in rhizoboxes.

nificantly higher ($P = 0.005$) in raw compared to steamed potting mix. *T. harzianum* did not have any effect on the total density of *P. ultimum* in either steamed or raw potting mix.

The microscopic examinations of the differentiation of *P. ultimum* as described below both refer to steamed and raw bulk potting mix and showed that 2 days after transfer of rhizoboxes with diseased plants, abundant mycelium was present a few centimeters from the roots. No globose structures (i.e., oogonia or oospores) were seen. After 4 days, abundant mycelium was still present a few centimeters from the roots. Past 4 cm, the presence of mycelium was very sparse. Globose structures, mostly oogonia, were observed. After 7 days, little mycelium was left. Newly formed oogonia, of which many were attached to hyphal fragments were found. After 14 days, the findings were similar to day 7. Zoospores were never observed.

Root-to-root spread of *P. ultimum*. There was no disease in the absence of oospores. There was no significant difference between the two experimental repetitions.

Both in steamed and raw potting mix, *P. ultimum* spread from infected plants roots to roots of noninoculated plants causing severe disease symptoms at all three distances (Table 2). No significant difference in disease severity was observed between steamed and raw potting mixes. Regression analysis showed that increased distances caused a significant decrease in disease severity ($r = -0.71$, $P < 0.001$). Likewise, the time required until the first of the noninoculated plant damped off increased by 2 days for each additional centimeter between adjoining rhizoboxes both in steamed and raw potting mix (Table 2). However, in raw compared to steamed potting mix, the time required for damping-off was delayed by 3 days for each of the distances.

Suppression of production and spread of secondary inoculum of *P. ultimum*. There was no disease in the absence of oospores. In steamed potting mix, seedlings inoculated with *P. ultimum* oospores damped off within 5 days (Table 3). The presence of *T. harzianum* within rhizobox one significantly ($P < 0.001$) reduced the disease severity on these inoculated plants. Compared to treatments without *T. harzianum* the reduction of the disease severity was 54% at day 5 and 7% at day 12.

The population densities of *P. ultimum* in the root zone of inoculated plants were undetectable at day 0 but increased throughout the experiment (Table 3). Both at day 5 and 12, the presence of *T. harzianum* within rhizobox one significantly reduced ($P < 0.002$) the population of *P. ultimum* compared to treatments without *T. harzianum*. If plants were inoculated with oospores in the absence of *T. harzianum*, and the rhizobox after 5 days transferred to, and thereby surrounded by potting mix infested with *T. harzianum*, the population density of the pathogen in rhizobox one increased more than twofold compared to the treatment without *T. harzianum*.

P. ultimum spread from roots of diseased plants in rhizobox one to roots of noninoculated plants in rhizobox two and caused severe disease symptoms 7 days after transfer of rhizobox one (i.e., day 12; [Table 3]). However, disease severity was significantly affected ($P < 0.001$) by the presence of *T. harzianum*. The presence of *T. harzianum* between the two rhizoboxes and around the roots of noninoculated plants in rhizobox two reduced the disease severity of these by 40%. When the primary infection was reduced by the presence of *T. harzianum* in rhizobox one, the secondary spread of *P. ultimum* was diminished and the amount of disease on noninoculated plants in rhizobox two was reduced by 79%.

The population densities of *P. ultimum* in the root zone of noninoculated plants in rhizobox two also responded significantly ($P = 0.027$) to the *T. harzianum* treatments (Table 3). Correlation analysis of treatments showed a similar reduction in population density of *P. ultimum* as in disease severity of plants in rhizobox two, when *T. harzianum* was added ($r = 0.71$, $P = 0.049$).

If oospores were added to rhizobox one in the absence of host plants, no disease symptoms could be observed on plants from rhizobox two (Table 3). Also, *P. ultimum* could not be recovered from either rhizobox.

The present work demonstrates that mycelial growth from infected plant tissue can be important as a means of spread of damping-off and root rot caused by *P. ultimum*. As for many other pathogen-inoculum sources, the concentration of the primary inoculum, the plant density, the distance separating diseased from healthy roots, the resident microflora, and the presence of an antagonist were shown to be important factors influencing disease spread by actively growing mycelium.

The importance of production and spread of secondary inoculum by *Pythium* spp. has been shown earlier (5,26). However, neither of these studies distinguished spread by mycelial growth from that of zoospores. Nor was spread through root contact excluded. As such, no clear evidence has, to our knowledge, previously been provided on the ability of *Pythium* spp. to spread actively through soil or potting mix by means of growing mycelium from primary foci to adjacent roots. The rhizobox system used in the present study confines roots thereby preventing contact between roots of plants grown in adjoining boxes. In this way, direct evidence for the spread of *P. ultimum* from diseased roots through bulk potting mix to roots of noninoculated plants was obtained. Microscopic examinations of the differentiation of *P. ultimum* var. *ultimum* in bulk potting mix confirmed that the spread was due to mycelial growth, as no zoospores were observed under these conditions. This is in accordance with van der Plaats-Niterink (28) who state that *P. ultimum* var. *ultimum* rarely produces sporangia. In addition, no movement of the artificially added oospores was observed, and as the oospore inoculum does not contain viable hyphal fragments, spread by other means than hyphal growth is unlikely to have occurred.

Judging from the population studies (Table 1), the density of mycelium dramatically decreased with increasing distances from the diseased roots. This is also illustrated by data in Table 2, where increased plant spacing caused a decrease in disease severity of noninoculated plants infected by the actively growing mycelium. The 2-day delay in the time required for damping-off to occur for each additional centimeter between inoculated and noninoculated plants is believed to reflect the time required by the mycelium to spread from plant to plant and to cause new infections. The resident microflora was found to have an adverse impact on the distance of spread and the rate by which it occurs (Tables 1 and 2). This reflects that *P. ultimum* belongs to the group of ruderals that is very sensitive to the presence of other competitive microorganisms (1,2,7).

When the pathogen was unable to reach a new host, production of terminal oogonia was observed. While the persistence of the

TABLE 2. Spread of *Pythium ultimum* (HB2) from diseased roots to roots of noninoculated plants grown in rhizoboxes as measured by disease index and days required until damping-off^a

Distance ^c	Disease index ^b		Days required until damping-off ^d	
	Steamed	Raw	Steamed	Raw
1 cm	4.00	3.89	4	7
2 cm	3.63	3.81	6	9
3 cm	2.69	3.22	8	11
LSD _{0.05}	0.76	0.27		

^a Data are based on two experimental repetitions. The experiments were carried out in steamed and raw potting mix.

^b The disease index relates to symptoms on roots and collar of noninoculated plants evaluated after 14 days. 0 = healthy looking, heavily branched root system; 1 = root system less branched than 0, light brown necrosis in distinct spots, often associated with the tip of side roots; 2 = only few side roots, and dark brown necrosis in distinct spots; 3 = few and small side roots, and dark brown necrosis of the entire root system, or all around the stem; 4 = plant dead.

^c By use of plastic-spacers, rhizoboxes with inoculated plants were placed at precise distances from rhizoboxes with noninoculated plants.

^d The time required until the first of the noninoculated plants damped off.

mycelium in potting mix is low, the oospores can act as survival structures for later establishment of root-pathogen contact. Where oospores are the primary inoculum the pathogen has very limited mobility, and thus root growth provides increased opportunity for root-pathogen contact (17). Once a successful infection has occurred, the pathogen can propagate on the infected root tissue and produce secondary inoculum, which can be spread to nearby roots. This study shows that both the concentration of the primary inoculum and the plant density are important for disease spread. In addition, oospore concentration and plant density interacted significantly (Fig. 3). The enhancement in disease severity due to increased seedling density was greatest at lower oospore concentration. This indicates that disease progression due to spread of the pathogen by active mycelial growth is more severe at high root densities. However, interpretations should be cautious as the root systems in dense plantings can be negatively affected thereby making them more susceptible to pathogen attack.

The inoculum used in this study did not include live mycelium but consisted of washed oospores which were almost entirely devoid of any external nutrient source. Extremely high oospore levels were necessary to obtain a consistent level of disease development. In addition, in raw potting mix, these oospores often failed to infect the test plants, and I. M. B. Knudsen (*unpublished data*), using oospore inoculum produced in the same way as in the present study, found that leaf debris buried in sterile soil was not colonized by *P. ultimum*. Several authors have been able to obtain a consistent level of disease using lower densities of inoculum. However, their inoculum either contained live mycelium (8,30) or an external nutrient source (3) that could support germination of oospores and mycelial growth. The present findings, therefore, indicate that the inoculum potential of pure oospores is very low. In contrast, probably due to the nutrient supply, mycelium growing actively from colonized plant tissue or debris exhibits a high inoculum potential as indicated in the present study and should therefore be considered as an important cause of disease establishment and progression. In accordance with this, it has been stated (4,13) that disease development due to *Pythium* spp. is better predicted on the basis of the saprophytic growth prior to infection than on the inoculum density.

This study suggests that the presence of *T. harzianum* can reduce the ability of *P. ultimum* hyphae to extend through bulk potting mix and cause disease on roots of adjacent plants (Tables 1

and 3). However, judging from the data in Table 1, this effect only seems to be valid in steamed potting mix. In raw potting mix, the effect of *T. harzianum* was undistinguishable from that of the indigenous microflora. Steaming of soils and potting mixes is known to release nutrients that can be utilized by microorganisms. We have previously shown that *T. harzianum* expresses increased metabolic activity during the first week after addition of a peat-bran formulation to steamed potting mix (12). Therefore, the recognition that growth and metabolism of *Trichoderma* must be active for successful biocontrol obviously also applies for the ability of *T. harzianum* to obstruct the spread of actively growing hyphae of *P. ultimum*. This may also explain why the population density of *P. ultimum* was much higher in the root zone of cucumber seedlings surrounded by steamed potting mix infested with *T. harzianum* compared to when the seedlings were surrounded by the noninfested mix (Table 1 and 3). At the time of transfer of the rhizoboxes, *P. ultimum* was well established on the seedlings, and the propagation on the infected root tissue could therefore be expected to be similar whether or not *T. harzianum* was present in the environment outside the rhizobox. As time elapsed, the obstruction by *T. harzianum* of hyphae extending from the primary foci resulted in an enhanced biomass of *P. ultimum* close to the roots compared to the situation without *T. harzianum*.

The presence of *T. harzianum* in the rhizosphere also reduced production of secondary inoculum of the pathogen on diseased root tissue. This is evidenced by the fact that *T. harzianum* reduced the population density of *P. ultimum* in the root zone of the plants whether inoculated by oospores or by actively growing mycelium (Table 3). A possible explanation is that the antagonist simply reduces the number of infections. Because fewer roots are successfully infected, there is less propagation by the pathogen, and thus less secondary inoculum is produced. Another explanation is that even though the roots are successfully infected, competition by *T. harzianum* for the infected and damaged root tissue (H. Green, N. Heiberg, K. Lejbølle, and D. F. Jensen, *unpublished data*) will reduce the availability of nutrients, consequently diminishing the multiplication and spread of the pathogen.

Concerning secondary inoculum produced by *Pythium* species, the focus is often on zoospores. But in the absence of an appropriate water potential, formation and discharge of zoospores are unlikely to take place, and even if they do, the mobility of the spores are severely restricted depending on the texture of the soil

TABLE 3. The influence of *Trichoderma harzianum* (T3) on the primary infection and subsequent spread of *Pythium ultimum* (HB2) between plants^a

Treatment ^b			Disease index ^c			Population density (CFU per gram of potting mix) of HB2 ^d		
			Box 1		Box 2	Box 1		Box 2
			Day 5	Day 12	Day 12	Day 5	Day 12	Day 12
Box 1	Between boxes	Box 2						
—	—	—	0	0	0	0	0	0
T3	T3	T3	0	0	0	0	0	0
HB2	—	—	4.00	4.00	3.42	10,884	34,168	2,232
HB2	T3	T3	4.00	4.00	2.04	10,884	78,304	1,445
HB2/T3	T3	T3	1.83	3.71	0.71	552	4,966	77 ^e
HB2 (–P) ^f	—	—	—	—	0	0	0	0
LSD _{0.05}			0.17	0.08	0.74	3,823	23,861	1,433

^a The experiment was repeated twice. Due to an incomplete design of the first repetition only results from the second repetition, which are representative of both, are presented.

^b Plants in the rhizoboxes were inoculated with different combinations of pathogen and antagonist. After 5 days when all plants inoculated with HB2 were severely diseased, the rhizoboxes were transferred to new pots. Plastic-spacers ensured a distance of 2 cm between the two rhizoboxes (Fig. 2). The pots were filled with additional potting mix either noninfested or infested with T3 corresponding to the treatment in rhizobox 2.

^c The disease index relates to symptoms on roots and collar of noninoculated plants evaluated after 5 and 12 days. 0 = healthy looking, heavily branched root system; 1 = root system less branched than 0, light brown necrosis in distinct spots, often associated with the tip of side roots; 2 = only few side roots, and dark brown necrosis in distinct spots; 3 = few and small side roots, and dark brown necrosis of the entire root system, or all around the stem; 4 = plant dead.

^d After 0, 5, and 12 days, the population density of HB2 in the root zone was measured by dilution plating and expressed as CFU per g of dry matter (potting mix).

^e The mean value was derived from two replicates. In spite of a considerably high variability in the methodology of dilution plating, the third replicate was disproportionately high (7,502 CFU per g) when compared to the two other replicates (75 and 78 CFU per g) and 10 other CFU values from the root zone of plants with a similar disease index.

^f No plants were grown in box 1. This control was included to test possible diffusion spread of oospores.

or growth medium (9). This study demonstrates that mycelial growth from diseased plant tissue also can be an important means of spread of damping-off and root rot caused by *P. ultimum*. Generally, compared to zoospores, the distance of mycelial spread from the primary foci is much restricted and obviously adversely affected by the resident microflora of the soil and potting mix. Thus, disease spread by mycelial growth is probably most important between roots of the same plant or adjoining plants and under conditions restricting the discharge and spread of zoospores. The presence of a metabolically active antagonist like *T. harzianum* may delay disease progression as production and spread of secondary inoculum is diminished.

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