

## The Role of Microbial Activity in Suppression of Damping-Off Caused by *Pythium ultimum*

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### ABSTRACT

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Addition of nutrients increased *Pythium* damping-off severity in a suppressive container medium amended with bark compost from the low temperature edge of a compost pile. A significant linear response of disease severity to added nutrients was observed. Levels of seed exudation were directly related to damping-off severity for seeds of a cucumber, a smooth-seeded, and a wrinkled-seeded pea cultivar. Container media amended with compost from the high temperature center of a compost pile (> 60 C) were conducive to the disease at first but became suppressive in 3 to 4 days after incubation at 25 C, as they became recolonized by mesophilic microorganisms. Initially concentrations of water extractable glucose, reducing sugars, and total carbohydrates in the center compost medium were higher than those in the edge compost medium. However, concentrations decreased to levels not significantly different from those in the edge compost medium after 1, 4, and 6 days, respectively. These reductions in carbohydrate concentrations coincided with an increase in suppression in the medium prepared with high temperature compost. This

increase with time was highly correlated with microbial activity, based on rates of hydrolysis of fluorescein diacetate ( $r = -0.919$ ,  $P < 0.001$ ), and also correlated with total biomass, based on extractable phospholipid phosphate ( $r = -0.509$ ,  $P < 0.003$ ). Populations of mesophilic bacteria, actinomycetes, and total fungi in the conducive high temperature compost-amended medium were significantly lower in the first 2 days after incubation at low temperature. After 4 days, populations had increased to levels similar to those in the medium amended with low temperature compost, and they stayed at that level thereafter. Percent infection of cucumber and germination of *Pythium* sporangia were significantly higher in the conducive than in the suppressive container medium. Infection of cucumber occurred before *Pythium* populations increased significantly. It was concluded that coexistence of large populations of mesophilic microorganisms, great microbial activity, low concentrations of available nutrients, and high degree of microbiostasis characterized container media suppressive to *Pythium* damping-off.

Propagules of *Pythium* spp. depend on exogenous nutrients for germination and ultimately for successful host infection (9,17,33). Seed and root exudates are the principal sources of organic carbon in nutrient impoverished soils. It has been postulated that rapid deprivation of exudates by soil microbes results in disease suppression (22). Green manures also may serve as nutrient sources and are rapidly colonized by *Pythium* spp., resulting in an increased inoculum density and consequently higher disease incidence (25,36). With time, however, amendment of soil with organic matter increases disease suppressiveness, presumably because of enhanced microbial activity (23,36). This occurs without a reduction of inoculum density of these pathogens (23,36).

A number of approaches have been used to examine changes induced by treatments affecting the nutrient status in soil. Where specific microorganisms involved in suppression are known, the commonly used procedure is to determine changes in their populations (14,25). Another approach is to measure soil microbiostasis, which is indicative of a nutrient sink in soil (15,22). Alabouvette et al (1) used yet another approach by monitoring changes in respiration rates in a *Fusarium* suppressive soil. Suppressiveness increased after an increase in total microbial activity induced by glucose amendment of the soil (1). Finally, Lumsden et al (24) found strong correlations between suppression of lettuce drop and soil dehydrogenase activity and other soil factors in soil amended with composted municipal sludge.

In this paper, we report that a new procedure developed to measure microbial activity in soil effectively predicted suppressiveness to *Pythium* damping-off in soilless container

media. Furthermore, changes in total biomass and soluble nutrient status in these container media were correlated to suppressiveness. Preliminary reports were published earlier (5,6).

### MATERIALS AND METHODS

**Container media.** Container media amended with composted hardwood bark (mostly *Quercus* spp.) were used throughout this study. This bark-wood mixture contains approximately 50% cellulose when fresh (11). Compost was prepared in a 2.2-m-tall pile, which was turned and mixed every 2 wk for 4 mo or more (4). The two container media consisted of Canadian sphagnum peat and perlite amended with low temperature (<40 C) and the high temperature (>60 C) composted hardwood bark removed from the edge or the center of a 4-mo or older compost pile, respectively. The volumetric mixing ratio was 2:3:5 for the peat, perlite, and composted bark, respectively. These two container media, hereafter are referred to as edge compost and center compost media. The edge compost medium is suppressive to cucumber damping-off caused by *Pythium ultimum* Trow, whereas the center compost medium is conducive immediately after it is formulated (4). Both container media were amended with lime as described before (4). Final pH ranged from 6.0 to 6.4. Controlled release fertilizer (Osmocote 14-14-14, Sierra Chemical Co., Milpitas, CA) was added to each container medium (15 g/L) immediately before bioassays were set up.

**Incubation time and disease suppression.** The edge and center compost media were incubated at 25 C in polyethylene bags (2 L per bag) for 0-7 days after all ingredients were combined. Cucumber (*Cucumis sativus* L. 'Straight Eight') seedling bioassays were performed at 20 C (4) at various time intervals after media

were prepared. Disease severity ratings were made 10 days after planting according to the following scale: 1 = symptomless, 2 = diseased seedling (either wilted or with visible lesions on the hypocotyl), 3 = postemergence, and 4 = preemergence damping-off. Treatments were replicated five times (five pots with eight seeds each per treatment), and the experiment was conducted with a completely randomized design. Mean disease severity of eight seedlings in each pot was calculated to present one replication.

#### **Addition of nutrients to the suppressive container medium.**

Sucrose and asparagine were dissolved in sterile distilled water and then added to the suppressive edge compost medium to final concentrations of 0.625–3.75 mg of sucrose and 0.125–0.75 mg of asparagine per gram dry weight of container medium. Cucumber bioassays were then set up immediately to evaluate the effect of added nutrients on suppressiveness.

**Effect of seed types and exudation on suppressiveness.** To determine the effect of seed types on damping-off severity, bioassays were set up as described above. Cucumber seeds and seeds of two pea (*Pisum sativum* L.) cultivars that differ in seed exudation were planted 1 cm deep in the edge compost and the center compost media and in a 1:1 (v/v) mixture of the edge and center compost media. The pea cultivars were Kriter (smooth-seeded) and Venus (wrinkled-seeded). Before planting, damaged and unusually colored seeds were removed from seed lots. Six seeds were used in each of five pots per seed type. Disease severities were determined and means were calculated as described above. Seed exudation was determined with a modified water soaking procedure (2). Instead of electrical conductivity produced within a 24-hr period in a water solution, we determined the amount of carbohydrate released because it correlates directly with susceptibility to damping-off (32). To estimate the amount of seed exudate, preweighed amounts of each seed type (30 cucumber seeds, 20 smooth-seeded Kriter pea seeds, and 10 wrinkled-seeded Venus pea seeds) were surface disinfested 3 min in 1% sodium hypochlorite and rinsed twice in sterile distilled water. These seeds were next soaked 24 hr at 25 C in 25 ml of sterile distilled water (four replicates per treatment). Thereafter, seeds were carefully removed from the water, and the remaining water volume was then measured. The concentration of total carbohydrates was determined in duplicate samples for each replicate with a phenol-sulfuric acid procedure (8). Glucose was used as a standard. Carbohydrate concentrations in the exudate were expressed as microgram glucose equivalents per seed and per gram of seed. Standard deviations were calculated for each seed type.

**Carbohydrate concentrations in container media.** Carbohydrate concentrations were determined in water extracts of noninfested container media after they had been planted with cucumber seeds. Cucumber seeds (eight per pot) or seedlings and roots were removed before extraction of media at designated time intervals. Each of four 10-g wet weight medium samples per treatment was added to 30 ml of precooled distilled water and shaken in an ice water bath (~0 C) for 3 min. Then the mixture was filtered through a filter paper into a centrifuge tube standing in an ice water bath. The filtrate was next centrifuged (10 min, 7,800 g) at 0 C. The supernate was filtered aseptically through a membrane (0.45  $\mu$ m) and stored at -20 C for up to 7 days until chemical analysis. Cold water filtered through filter paper was used as control to check for carbohydrate contamination in the procedure. Dry weights of container media were determined from duplicate 10-g samples collected at the time of water extraction.

Concentrations of glucose, reducing substances, and total carbohydrates in water extracts were determined with colorimetric methods. A glucose oxidase method was used to determine glucose concentrations (21) and reducing substances were measured by Nelson's method (27). A phenol-sulfuric acid method was employed to estimate total carbohydrate concentrations (8). D-Glucose was used as a standard for all three chemical analyses. Concentrations were expressed as per gram dry weight container medium.

**Microbial biomass and activity.** The microbial biomass procedure developed by White et al (37), based on phosphate concentration in total phospholipids, was used with minor

modifications. A 10-g container medium sample was placed in a phosphate-free detergent washed flask (four samples per treatment). Fifty milliliters of methanol, 25 ml of chloroform, and an appropriate amount of water, depending on the water content of the samples were added to give a ratio of 2:1:0.8. The mixture was shaken 2 hr (90 rpm, 25 C) in a flask sealed with a rubber stopper and then filtered through Whatman No. 1 filter paper under slightly negative pressure to remove container medium residues. The filtrate was transferred to a separatory funnel and additional chloroform and water were added to yield a final ratio of 1:1:0.9 for methanol, chloroform, and water, respectively. This mixture was shaken and allowed to settle overnight for phase separation. The chloroform phase was then eluted from the separatory funnel. The final chloroform volume was noted (usually 85–90% recovery) and stored at -20 C until lipid phosphate determination. To determine phosphate content in the lipid fraction, a known amount of the chloroform phase was placed into an acid-washed test tube, and the chloroform was evaporated at 40 C. Concentrated (35%) perchloric acid (1.2 ml) was next added to the dried lipids. The acid solution was then digested at 180–200 C in a sand bath. Digestion was carried out for about 2 hr until the solution became clear. Standards containing 0, 0.025, 0.05, 0.075, 0.1, 0.15, and 0.2  $\mu$ mol  $\text{KH}_2\text{PO}_4$  were heated in duplicate in a similar fashion. After cooling, phosphate content was determined with the colorimetric procedure described by Dittmer and Wells (7). Concentrations were estimated from a standard curve and expressed in micromoles of phosphate per gram of dry container medium.

Microbial activity was monitored by measuring the rate of hydrolysis of fluorescein diacetate (FDA) (Sigma Chemical Company, St. Louis, MO) as proposed by Schnurer and Rosswall (31). FDA is hydrolyzed by a number of enzymes in living cells, such as proteases, lipases, and esterases (31). The reaction yields water soluble fluorescein from water insoluble FDA. Five-gram container medium samples were placed in 250-ml flasks. Twenty milliliters of 60 mM potassium phosphate buffer (pH 7.6) was added. Reaction was started by adding 400  $\mu$ g of FDA (2 mg/ml of acetone). The reaction in one of the samples in each treatment was terminated at 0 time by adding 20 ml of acetone and served as a blank. The reaction mixture was shaken on a rotary shaker (90 rpm) at 25 C. A preliminary test showed that the reaction was linear up to at least 20 min. The reaction, therefore, was stopped after 20 min by adding 20 ml of acetone (31) with a Repipet dispenser. Container medium residues were removed from the mixture by filtration through a filter paper. The concentration of fluorescein was then determined by measuring absorbance at 490 nm. Standard curves were prepared as follows: 0, 100, 200, 300, and 400  $\mu$ g of FDA from the stock solution were added in duplicate to 5 ml of phosphate buffer in screw-capped test tubes. Test tubes were capped tightly and heated in boiling water for 60 min to hydrolyze FDA (31). Hydrolyzed FDA was then added to flasks containing 5-g compost samples. Another 15 ml of buffer was used to wash the hydrolyzed FDA down into the sample. Finally, 20 ml of acetone was added. The mixture was then filtered as described above. The absorbance was linear up to 300  $\mu$ g of FDA per sample, and absorbance of all treatments was within the linear range. A standard curve prepared by hydrolysis of FDA in a boiling water bath did not differ from that prepared with fluorescein (Sigma, St. Louis, MO).

To determine relationships of disease severity with microbial biomass, microbial activity, and specific activity (activity per unit of biomass) as well as the relationship of microbial biomass with activity, regression analyses were performed with a Minitab computer program. Regression lines and equations and correlation coefficients were presented with scattergrams.

**Microbial populations.** Populations of mesophilic fungi were determined on acid potato-dextrose agar supplemented with 1,000 ppm of Tergitol NP-10 (J. T. Baker Chemical Co., Phillipsburg, NJ) (sufactant-APDA) (15). *Trichoderma* spp. were isolated on a modified *Trichoderma*-selective medium (10 and see below); actinomycetes on an alkaline water agar (15) and fluorescent pseudomonads on King's B medium (19). Finally, copiotrophic

and oligotrophic (28) bacteria were determined on full-strength Difco nutrient agar (NA) amended with 100 ppm pentachloronitrobenzene (PCNB) (15) and on 100-fold diluted NA amended with 100 ppm PCNB, respectively. All media were sterilized by autoclaving and dispensed into 9-cm-diameter petri plates (15 ml per plate). The modified *Trichoderma*-selective medium (Chung and Hoitink, unpublished) contained 3 g of glucose, 1 g of  $\text{NH}_4\text{NO}_3$ , 0.9 g of  $\text{KH}_2\text{PO}_4$ , 0.2 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.15 g of KCl, 30 mg of rose bengal, 20 mg each of  $\text{FeSO}_4$ ,  $\text{MnSO}_4$ ,  $\text{ZnSO}_4$ , and 20 g of agar in 1,000 ml of distilled water. In addition, 50 mg of chloramphenicol, 50 mg of streptomycin sulfate, 0.1 g of PCNB, and 0.5 ml of metalaxyl were added after autoclaving. The modification reduced the time required for incubation from about 10 to 4 days.

At 2-day intervals up to 6 days after preparation of edge and center compost media, 10-g container medium samples (four replicates) were added to 90 ml of sterile 0.1% Difco water agar and homogenized (5 sec at full speed, three times with 30-sec intervals) with an Sorval Omni-Mixer (Newtown, CT). Further 10-fold dilutions were made with sterile water agar. Appropriate consecutive dilutions (three per dilution series) were plated in triplicate each (100  $\mu\text{l}$  per plate) and incubated at 25 C. Colonies were counted 48 hr after plating on NA and King's B media, 4 days after plating on surfactant-APDA and the *Trichoderma* selective media; 21 days after plating on dilute NA, and 28 days after plating on alkaline water agar. Colonies on King's B medium were examined under UV light. The mean number of colony-forming units (cfu) on triplicate plates was calculated to represent one replication. Microbial populations were expressed as colony-forming units per gram dry weight of container medium.

**Time of infection and *Pythium* population dynamics.** The cucumber seedling bioassay (eight seeds per pot) was set up with the edge and the center compost media as described above. Cucumber seeds or seedlings were recovered from each pot (five pots per treatment) at various time intervals from 0 to 10 days after planting. Recovered seeds or seedlings were washed with distilled water to remove container medium particles. They were surface disinfected (1 min) in 1% sodium hypochlorite, and rinsed with three changes of sterile distilled water. They were then placed on SA-PBNC *Pythium* selective medium and incubated for up to 4 days at 25 C. Infected seeds, as indicated by growth of *Pythium* mycelium on the selective medium, were removed aseptically with the surrounding colonized agar to prevent mycelial overgrowth on other seeds. Hyphal tips were isolated randomly and transferred to lima bean agar to verify pathogen identity as described before (4). The percentage of infected seeds in a pot was recorded to represent one replication. Each time that cucumber seeds were recovered, the container medium in each pot was mixed thoroughly. *Pythium* populations were determined on a 10-g sample from each pot by surface dilution plating on SA-PBNC medium (4). Ten percent of the colonies on the selective medium were randomly isolated and verified for *Pythium* identity as described before (4). *Pythium* populations were expressed as colony-forming units per gram dry weight of container medium. Dry weight was determined from a duplicate 10-g sample.

**Germination of sporangia in compost media.** Sporangia of *P. ultimum* were produced in a hemp seed broth and harvested as described by Schlub and Schmitthenner (30). Sporangia were collected on polycarbonate filters (8  $\mu\text{m}$  pore size) under negative pressure and washed (three times, 1 min each) with sterile double-distilled water. Sporangia on the filters (about  $10^5$  per square millimeter of filter surface area) were then placed on the edge and center compost media (at container moisture capacity) in petri plates and incubated at 25 C. After 4 hr, sporangia were stained with cotton blue in lactophenol and examined microscopically. Percent germination (germ tube longer than or equal to the diameter of the sporangium) was determined from three replicates of at least 100 sporangia each. The length of 10 randomly selected germ tubes was measured for each replication. Means and standard deviation were presented. Controls consisted of polycarbonate filters with sporangia incubated on APDA and WA.

## RESULTS

**Incubation time and disease suppression.** The edge compost medium was suppressive to *Pythium* damping-off (mean disease severity of 1.8) when it was first prepared and remained so throughout the test period (Fig. 1). The center compost medium was conducive (mean disease severity of 3.8) just after its formulation (0 time). However, disease severity decreased with time until after 3 days when it had declined to 1.6. At this time, disease severity was not significantly ( $P=0.05$ ) different from that in the edge compost medium (Fig. 1). Damping-off in the center compost medium was suppressed, thereafter. This experiment was repeated five times with two compost piles yielding similar results each time.

**Addition of nutrients to the suppressive container medium.** Addition of sucrose and asparagine to the suppressive edge compost medium rendered it conducive (Table 1). Disease severity increased as levels of added nutrients were increased. Regression analysis showed that the linear response of disease severity to addition of nutrients was significant ( $r=0.743$ ,  $P<0.01$ ). Addition of 3.75 mg of sucrose and 0.75 mg of asparagine per gram of edge compost medium increased the mean disease severity from 1.4 (without added nutrients) to 3.7. This level of disease was not significantly different ( $P=0.05$ ) from that in the conducive center compost medium. This experiment was performed twice and similar results were obtained.

**Effect of seed type and exudation on suppressiveness.** The edge compost medium suppressed damping-off caused by *P. ultimum* on cucumber and on both pea varieties. Disease severity values of the three hosts did not differ significantly ( $P=0.05$ ) (Fig. 2). The center compost medium, on the other hand, was conducive to the disease on all three hosts. In the container medium prepared with the 1:1 mixture of the edge and the center compost media, disease severity values for cucumber, smooth-seeded pea Kriter, and wrinkled-seeded pea Venus were 1.6, 2.4, and 3.6, respectively. These values were significantly different ( $P=0.05$ ) from each other (Fig. 2). Essentially the same results were obtained in a second experiment with these seed types and compost-amended container media.

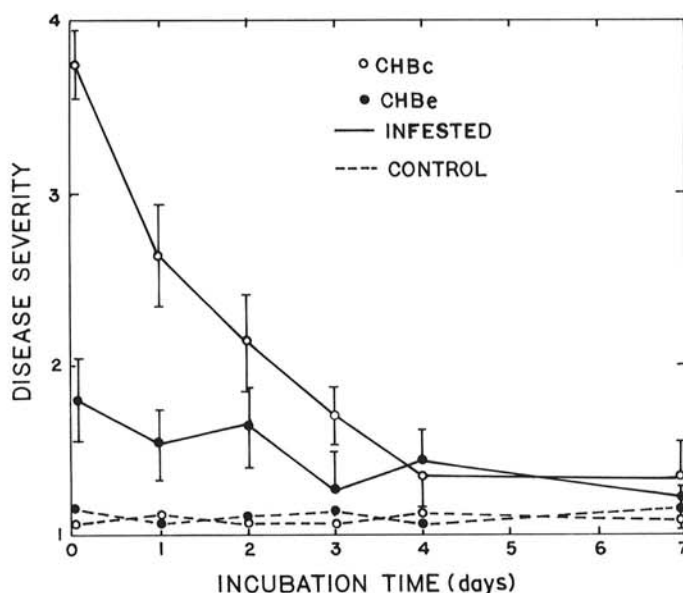


Fig. 1. Changes in suppressiveness of edge and center bark compost container media on incubation at room temperature. CHBe and CHBc represent container media amended with composted hardwood bark removed from the edge (< 40 C) or center (> 60 C) of compost piles, respectively. Solid line = infested with *Pythium ultimum* and dashed line = uninfested control. Disease severity rating scale: 1 = symptomless, 2 = diseased seedlings, 3 = postemergence, and 4 = preemergence damping-off. Each data point represents the mean of five replications of eight seedlings. Vertical bars represent standard deviation.

The three seed types differed significantly ( $P = 0.05$ ) in levels of seed exudation. The mean glucose equivalents for cucumber Straight Eight, smooth-seeded pea Kritter, and wrinkled-seeded pea Venus were  $7.1 \pm 0.79$ ,  $133.6 \pm 33$ , and  $402.9 \pm 79.9 \mu\text{g}$  per seed, respectively, or  $244 \pm 27.1$ ,  $1,131 \pm 279$ , and  $1,605 \pm 318 \mu\text{g}$  per gram of seed, respectively.

**Carbohydrate concentrations.** Concentrations of the three classes of carbohydrates initially were higher in the center than in the edge compost media, although patterns differed. The water extractable glucose concentration in the conducive center compost medium ( $120 \mu\text{g/g}$  dry wt) was higher than that in the suppressive edge compost medium ( $64 \mu\text{g/g}$  dry wt) (Fig. 3A). However, this difference disappeared within 24 hr. In both container media, glucose concentrations became essentially undetectable thereafter.

The concentration of reducing substances in the center ( $1.75 \text{ mg/g}$  dry wt) was higher than that in the edge compost medium ( $1.25 \text{ mg/g}$  dry wt) at 0 time (Fig. 3B). Significant differences persisted up to 3 days. Thereafter, they were not significantly different ( $0.25 \text{ mg/g}$  vs.  $0.09 \text{ mg/g}$  dry wt on day 4). At this time, the center compost medium became suppressive (Fig. 1).

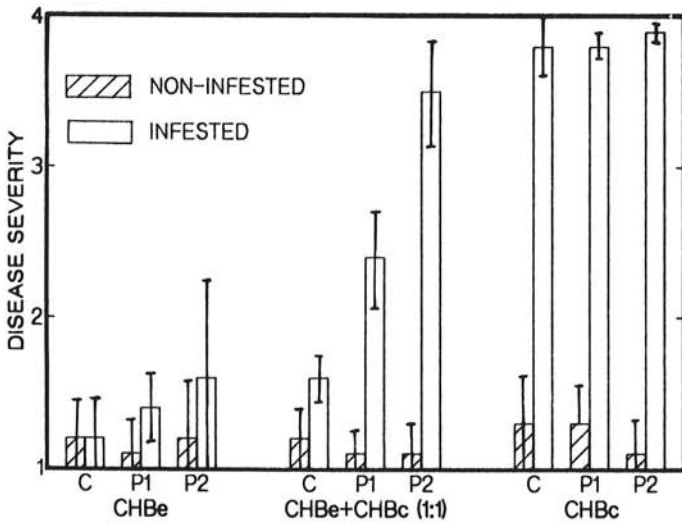


Fig. 2. Disease severity of cucumber cultivar Straight Eight (C), smooth-seeded pea Kritter (P1), and wrinkled-seeded pea Venus (P2) in a Canadian sphagnum peat and perlite container medium amended with compost samples removed from the low temperature edge (CHBe) or the high temperature center (CHBc) of a compost pile, and in a 1:1 mixture of CHBe and CHBc container media. Disease severities were determined according to the following scale: 1 = symptomless, 2 = emerged but diseased seedling, 3 = postemergence, and 4 = preemergence damping-off. Each bar represents a mean of five replications of six seedlings. Vertical bars represent standard deviation.

Concentrations of total carbohydrates in the two container media were not significantly different at 0 time. However, in the edge compost medium total carbohydrates decreased at a higher rate than in the center compost medium (Fig. 3C). Significant differences were found 1, 2, 3, and 4 days after planting (Fig. 3C). Similar results were obtained in a second experiment.

**Microbial biomass and microbial activity.** Immediately after container media were prepared (time 0), the microbial biomass (measured by phosphate concentration in total phospholipids) in the suppressive edge compost medium was significantly ( $P = 0.05$ ) higher ( $0.62 \mu\text{mol ppg}$  dry wt) than that in the conducive center compost medium ( $0.41 \mu\text{mol ppg}$  dry wt) (Fig. 4). There was no significant change in biomass in the edge compost medium during the next 7-day period. However, in the center compost medium, an increase in biomass was observed (an increase from  $0.41 \mu\text{mol ppg}$  to  $0.53 \mu\text{mol ppg}$  dry wt). On day 7, differences in microbial biomass between the two media were no longer significant (Fig. 4).

After container media were first formulated, microbial activity (measured by rate of FDA hydrolysis) in the edge compost medium was eight times higher than that in the center compost medium ( $4.2$  vs.  $0.5 \mu\text{g}$  of hydrolyzed FDA  $\text{min}^{-1} \text{g}^{-1}$  dry wt) (Fig. 5). It was 4.5 times higher after 1 day, and 1.3 times higher after 2 days. However, after 3 days, microbial activity in the center compost medium was 1.5 times higher than that in the edge compost medium. On days 4 and 5, microbial activity in both container media did not differ. On day 7, it was slightly higher in the edge than in the center compost medium (Fig. 5).

Regression analysis of all data from the edge and center media pooled together showed a correlation ( $r = -0.509$ ,  $P < 0.003$ ) between disease severity and microbial biomass (Fig. 6A). Disease severity was highly correlated ( $r = -0.919$ ,  $P < 0.001$ ) with microbial activity (Fig. 6B). Disease severity also was correlated ( $r = -0.837$ ,  $P < 0.01$ ) with specific activity (microbial activity per unit biomass) (Fig. 6C). Microbial biomass also was correlated with microbial activity ( $r = 0.455$ ,  $P < 0.01$ , not presented). Residual plots after regression analysis showed random distributions (not presented).

**Changes in microbial populations.** Significant differences in populations of mesophilic microorganisms between the edge and center compost media were present in the first 2 days after the container media were prepared. Microbial populations in the edge compost medium were higher than in the center compost medium and increased to some extent during incubation. In the center compost medium, population levels of mesophilic microorganisms were quite low at 0 time. With the exception of total fungi, population levels after 6 days in the originally conducive center compost medium had increased to or even exceeded total populations in the edge compost medium (Table 2). For instance, there was a 700-fold increase in mesophilic oligotrophic bacteria in the center compost medium (from  $2 \times 10^6$  to  $1.5 \times 10^9$  cfu/g dry wt) compared with only a 20-fold increase in the edge container

TABLE 1. Effect of added nutrients on suppressiveness of container media amended with bark compost to *Pythium* damping-off of cucumber

Container medium <sup>a</sup>	Added nutrients (units per g) <sup>b</sup>	<i>Pythium</i> inoculum <sup>c</sup>	Disease severity <sup>d</sup>
CHBc	0	—	$1.1 \pm 0.2$
CHBc	0	+	$3.8 \pm 0.4$
CHBe	0	—	$1.1 \pm 0.2$
CHBe	0	+	$1.4 \pm 0.8$
CHBe	1	+	$2.0 \pm 0.9$
CHBe	2	+	$2.0 \pm 0.9$
CHBe	3	+	$2.6 \pm 1.0$
CHBe	3	+	$3.6 \pm 0.4$
CHBe	6	+	$3.7 \pm 0.3$

<sup>a</sup> Container medium of Canadian sphagnum peat and perlite amended with samples of composted hardwood bark removed from the low-temperature edge (CHBe) or high-temperature center (CHBc) of a compost pile.

<sup>b</sup> One unit of nutrients contained 0.625 mg of sucrose and 0.125 mg of asparagine as a solution in sterile water.

<sup>c</sup> Infested with  $0.75 \text{ g}$  of soil inoculum of *P. ultimum* per liter of container medium.

<sup>d</sup> Disease severity ( $X \pm \text{SD}$ ,  $n = 5$ ) determined 10 days after planting on the basis of 1 = symptomless, 2 = diseased seedlings, 3 = postemergence, and 4 = preemergence damping-off. Based on five replicates of eight plants each. Regression analysis of the last six treatments showed that a linear response of disease severity to added nutrients was significant ( $r = 0.743$ ,  $P < 0.01$ ).

medium (from  $6 \times 10^7$  to  $1.3 \times 10^9$  cfu/g dry wt) in 6 days. On the other hand, there was only a fivefold increase in total copiotrophic bacteria in the edge compost medium and a more than 300-fold increase in the center compost medium (Table 2). There was a slight decrease in the population levels of actinomycetes in the edge compost medium. However, this population level in the center compost medium increased 100-fold. Whereas an 18-fold increase in population of total fungi occurred in the center compost medium (Table 2), no detectable change occurred in the edge compost medium. Similar trends were also found in population

development of *Trichoderma* spp. and of fluorescent *Pseudomonads*. In the center compost medium between days 4 and 6, after the container medium had become suppressive, there was a decrease or no increase in microbial populations. Similar results were obtained in a second experiment.

**Time of infection and *Pythium* population dynamics.** Percent infection of cucumber seedlings with time in the edge (suppressive) compost medium did not increase significantly ( $P = 0.05$ ) and was less than 5% throughout the 10-day bioassay period (Fig. 7A). In the center (conductive) compost medium, however, percent infection of cucumber seedlings increased with time. Percent infection reached 87.5% 4 days after planting and remained above 90% thereafter. Most of the infections occurred within the first 4 days (Fig. 7A).

The initial inoculum density in the center and edge compost media was approximately 60 cfu per gram dry weight. The *Pythium* population did not increase significantly ( $P = 0.05$ ) in the edge compost medium (Fig. 7B). On the other hand, in the center compost medium, the *Pythium* population began to increase significantly ( $P = 0.05$ ) after day 3. The most significant increase

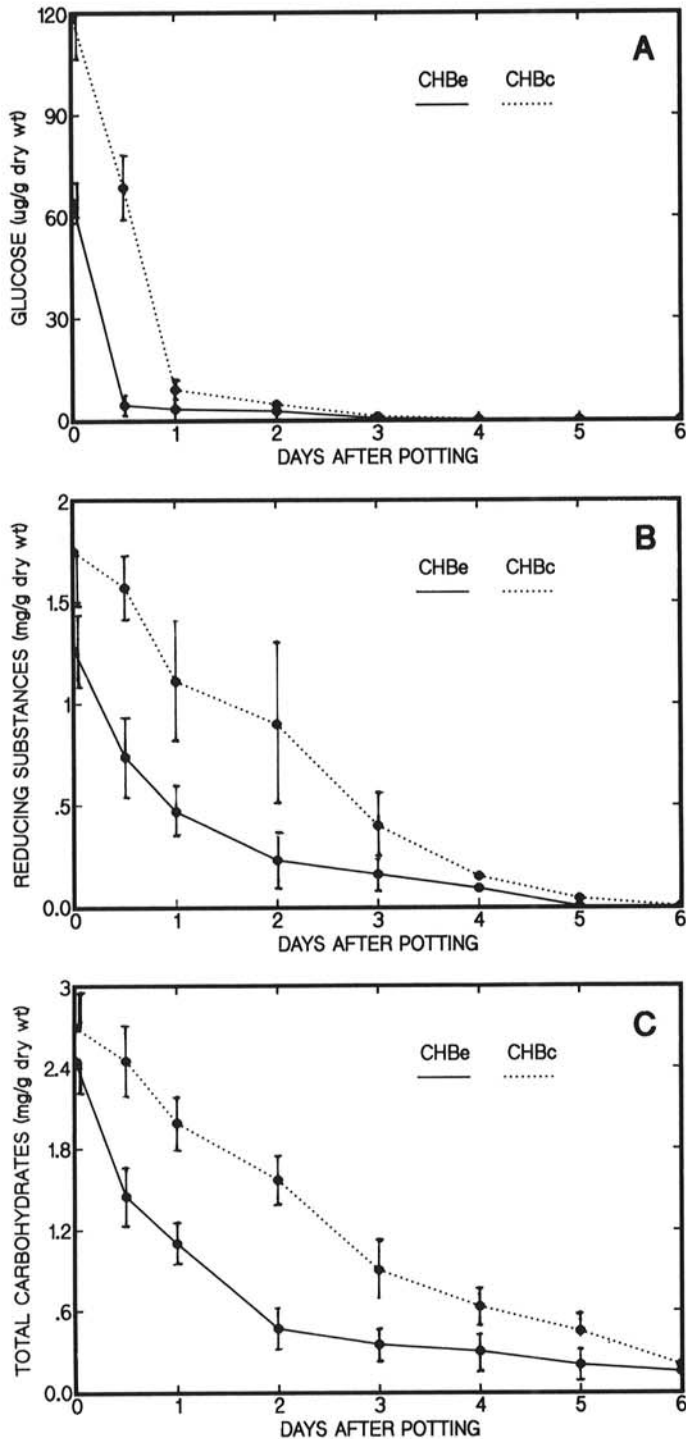


Fig. 3. Carbohydrate concentrations in bark compost container media with time after potting. CHBe and CHBc represent container media amended with composted hardwood bark removed from the edge (< 40 C) or the center (> 60 C) of compost piles, respectively. A, Glucose, B, reducing substances, and C, total carbohydrates. Vertical bars represent standard deviation ( $n = 4$ ).

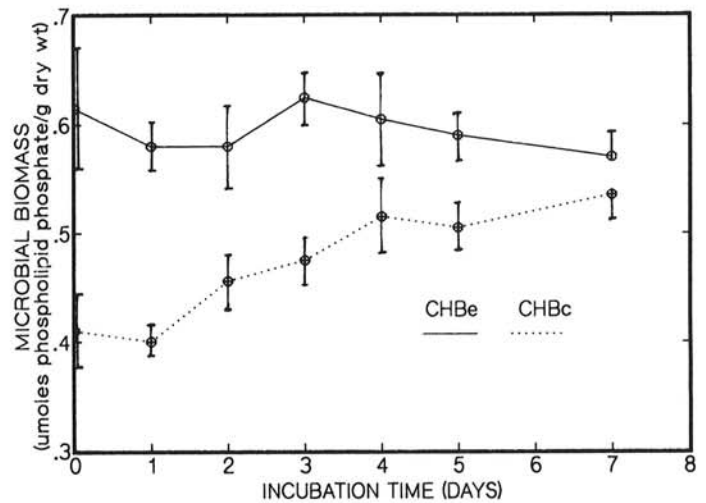


Fig. 4. Changes in total biomass as measured by extractible phospholipid phosphate in bark compost container media. CHBe and CHBc represent container media amended with composted hardwood bark removed from the edge (< 40 C) or center (> 60 C) of compost piles, respectively. Vertical bars represent standard deviation ( $n = 4$ ).

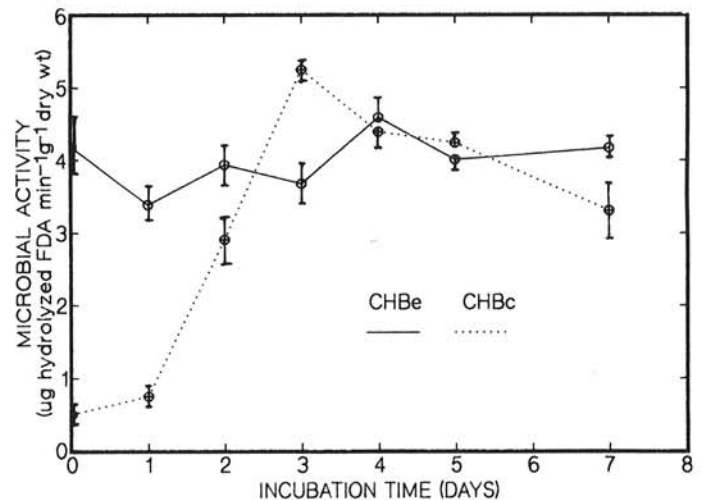
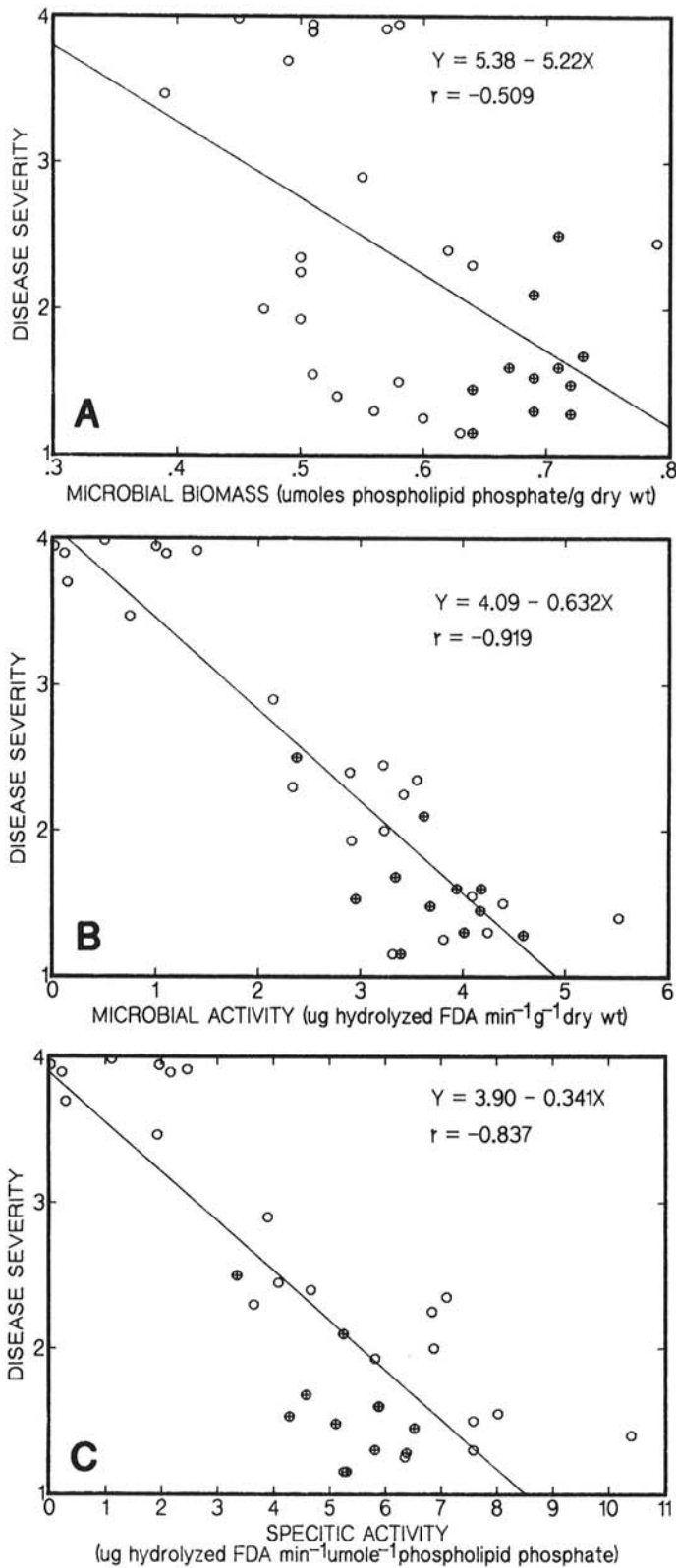


Fig. 5. Changes in microbial activity as measured by the rate of hydrolysis of fluorescein diacetate (FDA) in container media amended with composted hardwood bark removed from the center (CHBc) or the edge (CHBe) of a compost pile. Vertical bars represent standard deviation ( $n = 4$ ).

was observed between days 4 and 7 (Fig. 7B).

*Pythium* colonies on the SA-PBNC selective medium were



**Fig. 6.** Scattergrams showing the relationship of disease severity with, **A**, total microbial biomass based on extractable phospholipid phosphate, **B**, microbial activity as measured by hydrolysis of fluorescein diacetate (FDA), and, **C**, specific microbial activity (activity per unit biomass) in container media amended with composted hardwood bark removed from the high temperature (> 60 C) center (open circle) or the low temperature (< 40 C) edge (crossed circle) of compost piles. Each data point is the mean of five replications of disease severity and the mean of four replications for the X axis for each treatment.

isolated for identification. Ninety-four percent (129 out of 137 colonies) showed typical characteristics of *P. ultimum*. The remaining eight colonies produced *P. ultimum*-like sporangia, but no oospores were observed. They were considered to be *P. ultimum*. *P. ultimum* was not detected in noninfested container media.

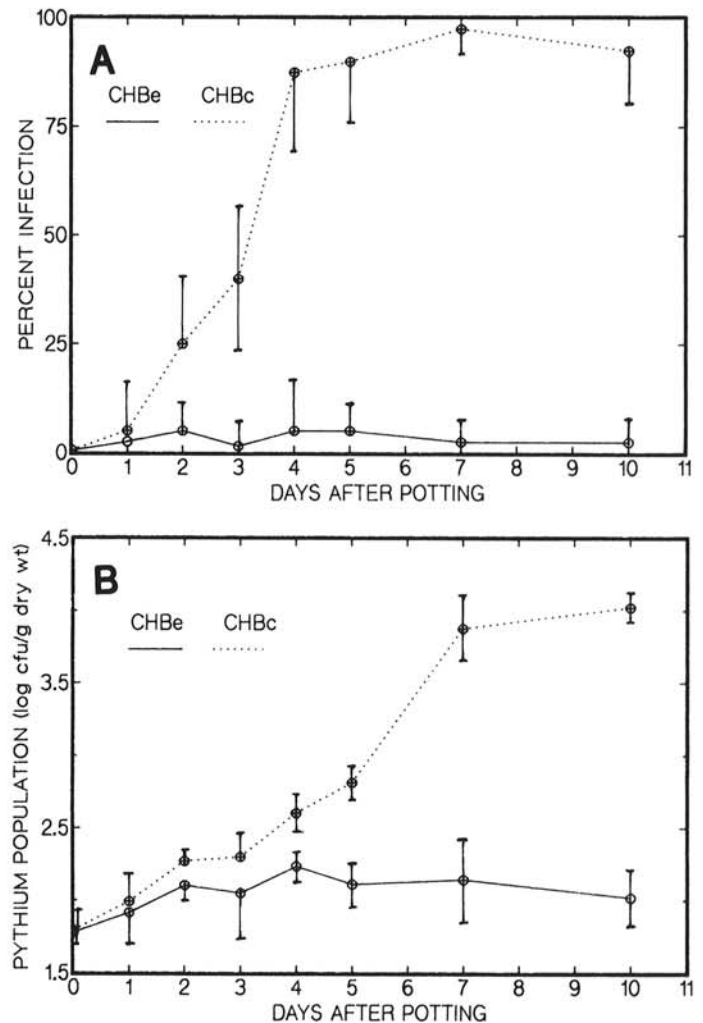
#### Germination of sporangia of *P. ultimum* in compost media.

Percent germination of sporangia on filters incubated 4 hr at 25 C on the center compost medium immediately after it was prepared (0 time) was the same as that on APDA (91%) (Table 3). Percent germination decreased with time. Three days after formulation of the center compost medium, only 35% of the sporangia germinated. In the edge compost medium, percent germination was only 24.3% at 0 time. After 3 days, it was as low as that in the WA control (8%). Similar percentages of germination were obtained in a second experiment.

Large differences in germ tube lengths between the center and edge compost media were found at 0 time (961 vs. 398  $\mu\text{m}$ ). After the container media had incubated 3 days, mean germ tube lengths were 176  $\mu\text{m}$  in the center compost medium and 111  $\mu\text{m}$  in the edge compost medium (Table 3).

## DISCUSSION

Container media amended with compost removed from the low temperature edge of compost piles, cured 4 mo or more, are



**Fig. 7.** Changes in percent infection of cucumber by *Pythium ultimum*, **A**, and in *Pythium* population, **B**, in a Canadian sphagnum peat and perlite container medium amended with compost samples removed from the low temperature (< 40 C) edge (CHBe) or the high temperature (> 60 C) center (CHBc) of a compost pile. Vertical bars represent standard deviation ( $n = 5$ ).

TABLE 2. Microbial populations (mean of cfu/g dry wt and standard deviation,  $n = 4$ ) in container media amended with composted hardwood bark removed from the higher temperature ( $>60$  C) center and the low temperature ( $<40$  C) edge of a compost pile and thereafter incubated at 25 C

Time (days) <sup>a</sup>	Oligotrophic bacteria <sup>b</sup> ( $\times 10^8$ )	Copiotrophic bacteria <sup>c</sup>			Fungi <sup>e</sup>	
		Total ( $\times 10^6$ )	Fluorescent ( $\times 10^5$ )	Actinomycetes <sup>d</sup> ( $\times 10^6$ )	Total ( $\times 10^4$ )	Trichoderma ( $\times 10^3$ )
Edge compost medium						
0	0.6 $\pm$ 0.2	6.1 $\pm$ 4.2	2.4 $\pm$ 0.4	6.3 $\pm$ 0.7	2.3 $\pm$ 0.6	1.3 $\pm$ 0.6
2	7.8 $\pm$ 4.2	36.4 $\pm$ 2.4	11.5 $\pm$ 1.8	4.5 $\pm$ 1.3	3.6 $\pm$ 0.8	5.6 $\pm$ 3.2
4	10.8 $\pm$ 3.8	47.8 $\pm$ 13.2	36.0 $\pm$ 2.8	3.3 $\pm$ 1.3	3.2 $\pm$ 0.9	9.6 $\pm$ 7.7
6	13.4 $\pm$ 4.0	28.4 $\pm$ 2.5	19.5 $\pm$ 3.3	4.5 $\pm$ 1.5	3.4 $\pm$ 0.8	9.6 $\pm$ 4.4
Center compost medium						
0	0.02 $\pm$ 0.01	0.2 $\pm$ 0.07	0.01 $\pm$ 0.00	0.1 $\pm$ 0.04	0.1 $\pm$ 0.05	0.14 $\pm$ 0.2
2	0.8 $\pm$ 0.5	11.3 $\pm$ 3.2	23.3 $\pm$ 4.1	2.1 $\pm$ 0.13	0.65 $\pm$ 0.3	1.5 $\pm$ 1.7
4	14.3 $\pm$ 7.1	77.3 $\pm$ 14.6	66.1 $\pm$ 13.7	11.9 $\pm$ 2.6	0.8 $\pm$ 0.4	3.0 $\pm$ 2.5
6	15.0 $\pm$ 5.9	67.9 $\pm$ 11.5	50.0 $\pm$ 13.0	7.8 $\pm$ 1.7	1.8 $\pm$ 0.5	9.7 $\pm$ 4.5

<sup>a</sup> Days after amendment of composted hardwood bark into container media.

<sup>b</sup> Oligotrophic bacteria were determined on 100-fold dilute nutrient agar amended with 100 ppm of PCNB.

<sup>c</sup> Total copiotrophic and fluorescent bacteria were determined on full-strength-nutrient agar amended with 100 ppm of PCNB and King's B media, respectively.

<sup>d</sup> Determined on alkaline water agar.

<sup>e</sup> Total fungi and *Trichoderma* were determined on APDA amended with 1,000 ppm of Tergitol NP-10 and a *Trichoderma*-selective medium, respectively.

TABLE 3. Percent germination and germ tube length ( $\mu\text{m}$ ) ( $\bar{X} \pm \text{SD}$ ,  $n = 3$ ) or sporangia of *Pythium ultimum* in container media amended with bark compost removed from the higher temperature center (CHBc) or low temperature edge (CHBe) of a compost pile

Container medium	Time <sup>a</sup> (days)	Germination <sup>b</sup> (%)	Germ tube length <sup>c</sup> ( $\mu\text{m}$ )
CHBc	0	91.7 $\pm$ 0.6	961 $\pm$ 35
	1	55.7 $\pm$ 3.9	165 $\pm$ 23
	3	35.1 $\pm$ 9.3	176 $\pm$ 15
CHBe	0	24.3 $\pm$ 10	398 $\pm$ 53
	1	11.6 $\pm$ 2.0	110 $\pm$ 9
	3	8.6 $\pm$ 2.8	111 $\pm$ 8

<sup>a</sup> Days of incubation (25 C) of container media after their preparation.

<sup>b</sup> Determined 4 hr after incubation based on at least 100 sporangia per replication. Percent germination in controls were: 91% on acid potato-dextrose agar and 8% on water agar.

<sup>c</sup> Determined 4 hr after incubation based on 10 germ tubes per replication.

suppressive to cucumber damping-off caused by *P. ultimum* (4) and *P. aphanidermatum* (12). On the other hand, container media prepared with samples removed from the high temperature center are conducive (4,12,16). Suppression of the disease caused by *P. ultimum* is related to suppression of population development of this pathogen and it occurs without a reduction in inoculum density (4 and Fig. 7B). We proposed, therefore, that antibiosis or exploitation were not the principal mechanisms responsible for suppression, but that microorganisms in the suppressive medium were actively taking up nutrients creating a nutrient sink (4). Destruction of the suppressive effect in the edge compost medium by added nutrients (Table 1), the presence of higher concentrations of soluble carbohydrates in the conducive than in the suppressive container media (Fig. 3 A-C), and the direct relationship between seed exudation and damping-off severity all suggest that suppression indeed was due to lack of availability of nutrients for germination of propagules of *P. ultimum* and for infection of cucumber by *P. ultimum*. This is in agreement with previous reports on effects of added nutrients and fresh plant debris on disease severity caused by *Pythium* spp. (3,17,25). Disease severity observed with the three seed types in the three container media (Fig. 2) support an earlier finding that the level of seed exudation relates directly to damping-off severity (32). Data in Figure 2 also show that expression of this relationship between disease severity and seed exudation is dependent on the suppressiveness of the container medium.

Changes in microbial populations are indicators of the physiological status microorganisms and of the nutritional status

of their environment. A decrease or small increase indicates the presence of growth limiting factors and is a characteristic of microbiostasis (15). In the absence of growth limiting factors, a logarithmic increase results. Microbial populations in the suppressive edge compost medium were under more or less static growth conditions (Table 2). Furthermore, Kwok et al (20) showed previously that introduction of spontaneous rifampicin-resistant mutants of specific bacterial antagonists into the edge compost medium does not result in an increase of their populations. On the other hand, the bacteria extensively colonized cucumber roots in the conducive compost medium as well as the container medium itself. This shows that competition is severe and that microbiostasis prevails in the suppressive medium. *P. ultimum* introduced into this container medium, therefore, also was subjected to microbiostasis. For example, germination of sporangia of *P. ultimum* was significantly lower in the suppressive than in the conducive medium (Table 3). Reduced germination and reduced growth of *P. ultimum* explain the lower disease severity. Thus fungistasis resulted in suppression of *Pythium* damping-off in the compost-amended container media (sensu Lockwood, 22).

Our data on microbial activity in compost-amended container media, as determined with the FDA hydrolysis procedure, were comparable to findings of McKinley and Vestal (26) for compost proper. They found that incubation at low temperature of compost samples removed from the high temperature center of piles results in relatively low rates of incorporation of acetate into microbial lipids. On the other hand, samples removed from the low temperature edge have high microbial activity. Furthermore, they found that if both samples are incubated at high temperature, which is optimal for thermophilic microorganisms (34), center composts have higher microbial activity than edge composts (26). Therefore, the low microbial activity in the center compost medium in our work was due to the shift of temperature from high ( $>60$ ) to low (25 C). Finally, the low microbial activity in the center compost medium explains the high levels of carbohydrates that accumulated in that medium (Fig. 3A-C). After mesophiles had recolonized the biological vacuum (Table 2), microbial activity increased and carbohydrate concentrations were reduced to low levels, rendering the container medium suppressive (Figs. 2 and 3).

The close similarity in data obtained with FDA hydrolysis (Fig. 4) and incorporation of acetate into microbial lipids (36) and the correlation between FDA hydrolytic activity and respiration in soil samples from different layers of an agricultural soil (31) suggest that FDA hydrolysis is an indicator of microbial activity. This microbial activity assay is simple, rapid, and sensitive (31). Therefore, this method may prove useful in comparative studies of the phenomenon of general suppression and of soil microbiology

in general.

Comparison of nutrient quantities added to the suppressive edge compost medium (Table 1) with the differences in carbohydrate concentrations present in the conducive and suppressive compost media (Fig. 3C) shows that higher concentrations were required to render the suppressive edge compost medium conducive than the amount of carbohydrates that accumulated early after formulation of the center compost medium. The nutrient-starved microorganisms in the edge compost medium probably were able to use the added nutrients before sporangia of *P. ultimum* could germinate and infect cucumber seedlings. Therefore, the observed suppression was not merely due to the low levels of carbohydrate concentrations present. Rather the low carbohydrate concentrations reflected high microbial activity in the edge compost medium.

Extractible carbohydrates in the conducive compost medium were adequate in concentrations for population development of mesophilic microorganisms in the container medium, thus rendering it suppressive. However, this source of nutrients is not adequate to support *Pythium* population development. We showed previously that *Pythium* population did not increase in the unplanted conducive compost medium (4). Apparently the source of nutrients most important for development of the *Pythium* population were exudates and the host plant proper. Data in Figure 7A and B clearly show that infection of cucumber preceded a significant increase of *Pythium* population. Furthermore, as mentioned above, the higher the level of seed exudate, the higher the disease severity. The foregoing all suggest that suppression of damping-off caused by *P. ultimum* in compost-amended container media largely depends on interactions among this pathogen, other microorganisms, and seed exudates in the spermosphere.

In this work, we demonstrated a high correlation between microbial activity, as measured by FDA hydrolysis, and induction of suppression to damping-off caused by *P. ultimum*. To our knowledge, this is the first report for such an in situ analysis for this disease. High microbial activity induces intense microbial competition. *Pythium* is known as a pioneer colonizer but is a poor competitor because it is not able to colonize precolonized substrates (25,29). Under conditions of intense competition, saprophytic and pathogenic activities of *P. ultimum* are limited, resulting in lowered disease severity. This appears to be the mechanism of suppression of *P. ultimum* in the bark compost medium.

The low correlation between disease severity and biomass ( $r = -0.509$ ) and between biomass and activity ( $r = 0.455$ ) observed in this work can be explained as follows. Thermophiles, the predominant microbial community in the high temperature center compost, are replaced by mesophiles as the temperature of center compost samples decreases (34). The assay for microbial biomass used here cannot differentiate biomass of mesophiles from that of thermophiles. Thermophiles were not active at low temperature. Therefore, the FDA hydrolysis assay only measured activity of mesophiles.

Temperature is an important factor in the development of *Pythium* diseases. The optimum temperature for *P. ultimum* in raw soil is lower than in pure culture (13), suggesting that *P. ultimum* is less affected by the decrease in temperature than other soil microbes. Diseases caused by *P. ultimum* usually occur at low temperature (35) and in poorly drained soil (18). Several mechanisms have been proposed to explain this phenomenon. One is that low temperature increases plant exudation. Another is that low temperature reduces plant growth rates and that this increases opportunity for infection by *P. ultimum* (35). Thirdly, it has been proposed that *P. ultimum* can tolerate low oxygen tensions and that high moisture content increases plant exudation yielding more nutrients in the infection court (18). A fourth explanation can be inferred from this study. Sudden drops in temperature and/or oxygen tensions may drastically reduce total microbial activity in soil. Consequently, saprophytic competition is reduced. Thus more nutrients can become available to a nutrient dependent pathogen, such as *P. ultimum* that is less affected by this unfavorable environmental change. The result is increased disease severity.

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