An In Vitro System for Studying the Effects of *Pythium ultimum* Metabolites on *Pelargonium* × *hortorum*

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ABSTRACT

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An in vitro bioassay was developed to investigate the toxicity of metabolites of *Pythium ultimum* on geranium (*Pelargonium* × *hortorum*). Unrooted plantlets from two geranium cultivars were subcultured on a rooting medium supplemented with 2.5% (v/v) culture filtrates of *P. ultimum*. After 10 days of growth on the treated medium, the emerging roots of the plantlets appeared thinner, shorter, and darker than the controls. Several necrotic lesions developed on the root surface, and microscopic observations showed an absence of root hairs and a collapse of cortical and epidermal cells. In addition, vegetative growth of plantlets was stunted for both cultivars, even in the absence of foliar symptoms. Symptoms observed in vitro were similar to those associated with infections of *P. ultimum* on the same cultivars in the greenhouse. The in vitro sensitivity of the cultivars to the fungal metabolites also was related to their susceptibility to the pathogen. This study supports the hypothesis that *P. ultimum* metabolites are involved in the pathogenicity on *P.* × *hortorum*.

Pythium spp. are the most important cause of the pre- and postemergence phases of damping-off (8). Although *Pythium* spp. are usually considered pathogens of younger plants, they can also cause severe root and stem rot of mature plants (12). For instance, vegetable and bedding plant species such as cucumber (*Cucumis sativus* L.) (6), spinach (*Spinacia oleracea* L.) (21), lettuce (*Lactuca sativa* L.) (20) and geranium (*Pelargonium* spp.) (7,22) are susceptible to *Pythium* attacks at any stage of their production. This problem has been especially important in hydroponically grown cultures in which the fungus can spread easily (26).

Despite the importance of *Pythium* diseases, no commercial varieties resistant to this pathogen are available or have even been described, because of the limited knowledge of the host-*Pythium* interaction. Only a few investigators have studied the potential host defense mechanisms against *Pythium* (3,25), or the mode of action of the pathogen (22).

A few authors have suggested that *Pythium* spp. can produce toxic metabolites that play a role in a pathogenic interaction (4,11,23). While Ichihara et al (10) characterized one phytotoxic compound produced by *Pythium ultimum* Trow, Mojdehi & Singleton (15,16) reported that a complex of metabolites may be produced by *Pythium* spp. However, the biological role of these metabolites has never been characterized in a host-*Pythium* interaction. In view of the need to develop resistant or tolerant varieties to *Pythium*, the use of these metabolites in a bioassay to evaluate plant response and understand defense mechanisms could be valuable.

There are several examples in which undifferentiated calli or cells in vitro were used in bioassays with toxic metabolites or culture filtrates isolated from a pathogen (5). Bélanger et al (1) proposed an in vitro bioassay that involved inoculation of leaves and stems of plantlets of *Populus tremuloides* Michx. with toxic metabolites of *Hypoxylon mammatum* (Wahl.) Mill. In the present study, we introduce the use of plantlets at the rooting stage in vitro and the incorporation of fungal metabolites into the culture medium of the host for studying the role of metabolites of *P. ultimum* on *Pelargonium* × *hortorum* L. H. Bailey.

MATERIALS AND METHODS

Tissue culture of *Pelargonium*. Hybrid-seed geranium plants, (*Pelargonium* × *hortorum* 'Orbit White' and 'Hollywood Red') from Jack Van Klaveren Ltd., St. Catharines, Ont., Canada, were grown in Pro-Mix (Premier, Rivière du Loup, Qc, Canada) as mother plants under greenhouse conditions. Axillary meristems were taken from 10-wk-old plants, surface-sterilized in 0.5% sodium hypochlorite for 10 min, and rinsed three times in double-distilled water. Explants were then cultured on a modified (0.5 × macrosalts) Murashige and Skoog (MS) (17) basal medium (Sigma Chemical Co., St. Louis, MO.) supplemented with 0.02 mg of naphthaleneacetic acid (NAA) and 0.2 mg of 6-benzylaminopurine (BAP) per liter and containing 3% sucrose and 0.8% agar. The pH of the medium was adjusted to 5.7. Culture conditions were maintained at 23 C under cool-white fluorescent lights with an irradiance of 25 μE m^{-2} sec^{-1} for a photoperiod of 16 hr per day. The first shoots appeared after a few weeks. Plantlets were then subcultured on a fresh medium every 2 wk until a sufficient number of plantlets was obtained.

***Pythium* culture filtrates.** The isolation of the fungal filtrates was based on the methods described by Vidhyasekaran et al (24). *P. ultimum*, BARR 447 (Biosystematics Research Institute, Ottawa, Ont., Canada), was grown on Difco potato-dextrose agar (PDA) on 9-mm petri dishes for 3 days. Five 10-mm discs of actively growing mycelium were used to inoculate 200 ml of Difco potato-dextrose broth (PDB) in 500-ml flasks. For the controls, five discs of sterile PDA were used to inoculate 200 ml of PDB and the broth was subsequently treated exactly the same way as the *Pythium* filtrates. After 20 days of growth on a rotary shaker at room temperature, the culture broth was filtered through Whatman No. 1 paper, frozen at −80 C, and lyophilized. The culture filtrates were then dissolved in 50% methanol, stored overnight at 5 C, and filtered (Whatman No. 1) to remove precipitates. After evaporation in vacuo to dryness, the final volume was adjusted to 2% (v/v) of the original culture filtrate volume with 80% methanol. These partially purified culture filtrates were filter-sterilized with 0.22-μm membranes and used for the bioassays.

Bioassay. A volume of 0.5 ml of a partially purified culture filtrate of *P. ultimum* or of a control filtrate was added to 1 L

of reduced MS medium (0.5 × minerals), without NAA or BAP amendments, suitable for rapid rooting of in vitro geranium plantlets (H. Desilets, *unpublished*). This concentration corresponded to 2.5% (v/v) based on the crude filtrates volume. The filtrates were added to the autoclaved medium before the agar solidified. The medium then was dispensed in 20-ml aliquots to 25- × 100-mm test tubes. Unrooted geranium plantlets from in vitro clones were transferred onto these media and maintained under growth conditions as previously described. The experimental unit was composed, for each cultivar and for media treated with *Pythium* filtrates and control filtrates, of 10 plantlets each placed in individual tubes. This experiment was repeated twice.

Plantlets were observed daily for symptoms. Fresh weight, root diameter, and root number of plantlets were measured after 20 days of growth. Root samples (2 cm below the base of the stem) also were collected (one test tube for each treatment) after 10 days, and prepared for microscopic observations. They were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 24 hr at 4 C, and postfixed with 1% osmium tetroxide in the same buffer for 1 hr at room temperature before being dehydrated in an ethanol series. Samples for scanning electron microscopy were dried at critical point and were gold-coated. Samples for light microscopy were embedded in EPON 812. Transverse sections were observed under phase-contrast light microscopy after being stained for 2 min with toluidine blue (0.96 mg/ml).

Greenhouse inoculations. To compare results from the bioassay to in vivo conditions, 30-day-old plants from both cultivars were each inoculated with four mycelial disks of *P. ultimum* BARR 447 taken from 3-day-old cultures. Sterile PDA disks were used for controls. Ten plants of each cultivar were inoculated in both cases. After inoculation, the plants were transferred to a greenhouse where day and night temperatures were 22 and 19 C, respectively. Supplemental lighting (100 μE m^{-2} sec^{-1} PAR) was supplied by high-pressure sodium lamps for a 16-hr photoperiod. Disease symptoms and dead plants were recorded daily for 8 wk. At the end of that period, plants were oven-dried and weighed. Results obtained from these inoculations were part of an experiment done by Chagnon (2).

RESULTS

Bioassay. All plantlets were free of symptoms until emergence of the roots, which occurred 7–10 days after their transfer onto the rooting media. The roots growing in the medium that contained filtrates of *P. ultimum* appeared thinner and more fragile than the controls. Their surfaces were irregular and dark, and several localized necrotic lesions were present (Fig. 1A, see arrow). In several instances, at the site of lesions, subsequent growth was characterized by abnormal development. Roots of treated plantlets were also shorter than the controls (Fig. 1B). Root hairs were rare or absent, while they were abundant on roots of plantlets grown in the medium treated with the control filtrates (Fig. 2).

When compared to roots of the controls (Fig. 3A), sections taken from the roots with developing lesions showed destruction of epidermal and cortical cells (Fig. 3B, see arrow). Similar alterations were also observed along the entire length of the root, but to a lesser degree. The stele and the endodermis of roots appeared normal in plantlets grown in both the control and in the presence of culture filtrates of *P. ultimum*. However, thickening of the cell wall in the Casparian strip was very prominent in damaged roots (Fig. 3B, see double arrow).

Plantlet growth was less on the medium treated with culture filtrates than on the medium treated with control filtrates (Fig. 4A). After 20 days, the average weight of plantlets of both cultivars was lower than the controls, with a reduction of 35% for plantlets of cv. Orbit White. The mean root diameter was also reduced as a result of the treatment for both cultivars, with average values that dropped from 37 to 34 mm for Orbit White and from 38 to 32 mm for Hollywood Red. Interestingly, the average number of roots per plantlet was not affected on plantlets of Orbit White (5.9 vs 5.4), yet almost doubled on Hollywood Red plantlets (4.9 vs 9.0). Over the 20-day period, the treatment with culture filtrates of *P. ultimum* never caused plantlet mortality.

Greenhouse inoculations. Only five cases of mortality were recorded after inoculations with *P. ultimum*, and four of them were recorded on plants of Orbit White. No control plants died. Three weeks after inoculation, symptomatic leaves of surviving plants dried up and fell off and plants resumed active growth. Many plants appeared totally healthy at week 4, despite the presence of *P. ultimum* in their root systems. At the end of the experiment, surviving plants showed little or no apparent disease symptoms, but growth and flowering were delayed compared to the controls and their root systems were thin and poorly developed. At the end of the experiment, dry weights were lower for inoculated plants of both cultivars than for the controls. As in the bioassay, treated plants of Orbit White suffered more yield loss than those of Hollywood Red (Fig. 4B).

DISCUSSION

The use of toxic metabolites in the form of fungal filtrates or purified toxins has contributed to the understanding of several

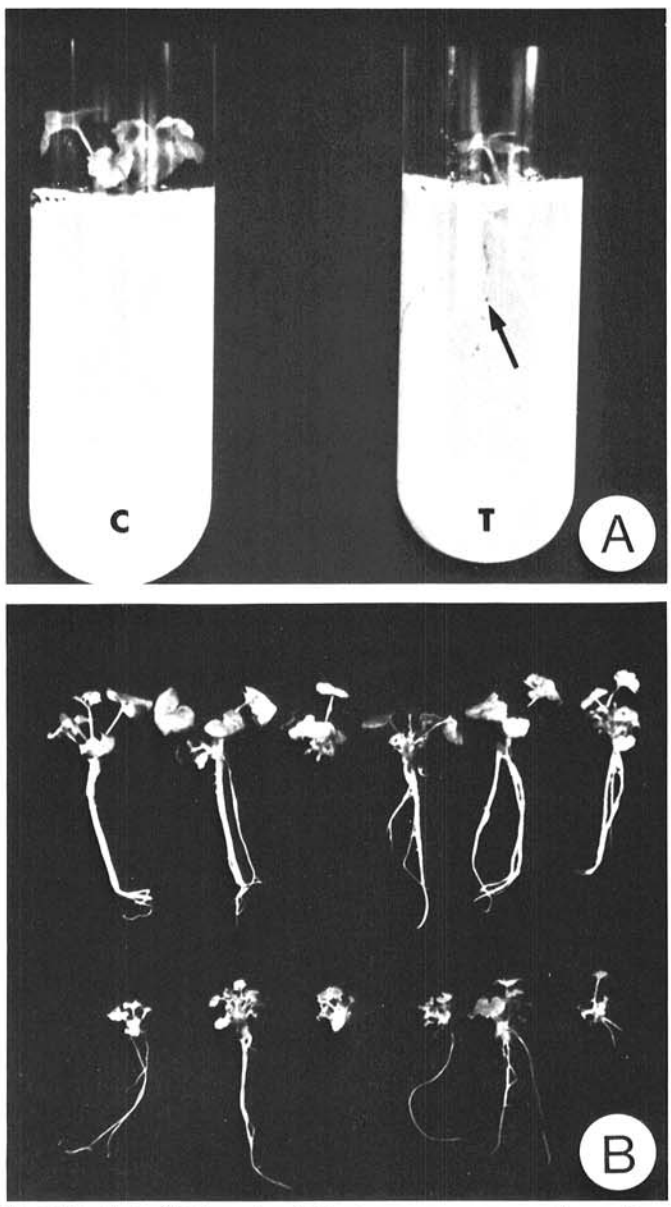

Fig. 1. Plantlets of *Pelargonium* × *hortorum* grown on a rooting medium treated with control filtrates (C) or 2.5% (v/v) culture filtrates (T) of *Pythium ultimum*. **A,** After 10 days of growth (arrow indicates necrotic lesions). **B,** After 20 days of growth. Control plantlets are in the upper row in **B**.

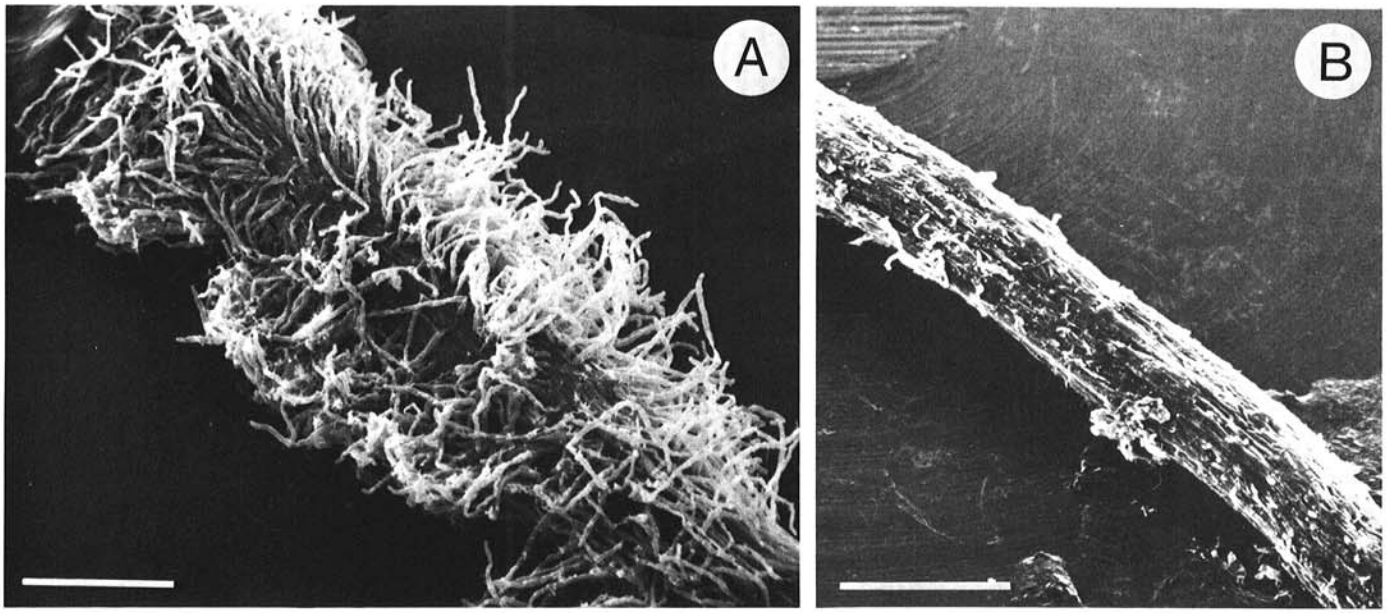

Fig. 2. Scanning electron micrograph of roots of *Pelargonium* × *hortorum* plantlets after 10 days of growth. **A,** Growth on a rooting medium treated with control filtrates. **B,** Growth on a rooting medium supplemented with 2.5% (v/v) culture filtrates of *Pythium ultimum*. Scale bars represent 40 μm.

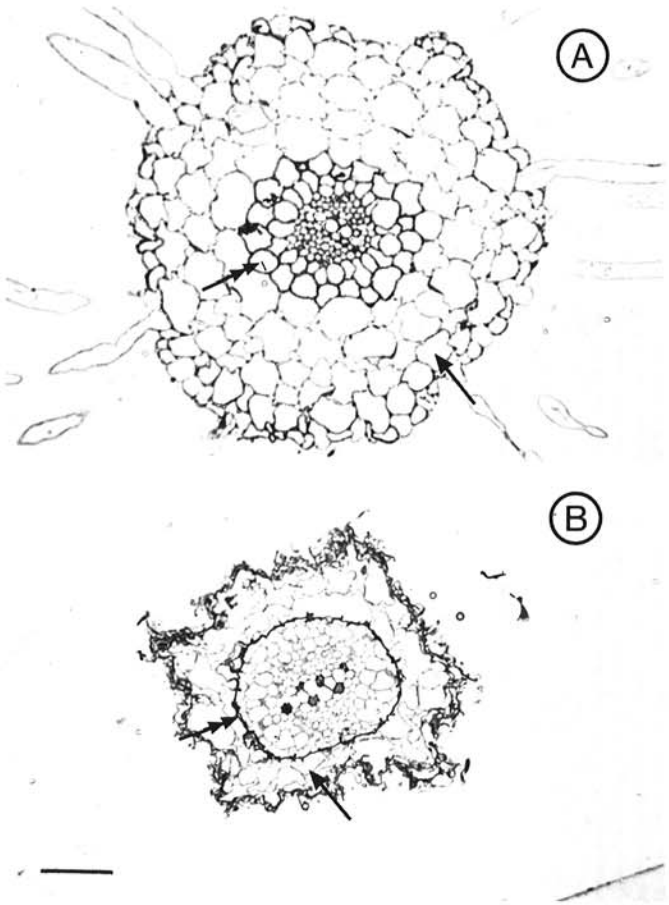

Fig. 3. Transverse sections of roots of plantlets of *Pelargonium* × *hortorum* after 10 days of growth. **A,** Growth on a rooting medium treated with control filtrates. **B,** Growth on a rooting medium supplemented with 2.5% (v/v) culture filtrates of *Pythium ultimum* (arrow indicates the cortical zone and double arrow the endodermis). Scale bar represents 50 μm.

host-pathogen interactions (5). However, in some instances, host response to the toxin was not correlated with pathogenicity (1,19). This discrepancy was sometimes attributed to the testing of the filtrates on undifferentiated tissues such as calli or cells (14). *P. ultimum* is a soilborne pathogen that mainly affects roots and rootlets of its hosts (8), and thus development of a bioassay for the rooting stage of in vitro plantlets appeared logical. This bioassay, which involved addition of low concentrations of culture filtrates of *P. ultimum* into the rooting medium, resulted in marked morphological and cytological alterations of roots and growth reduction of the plant material. This suggests that *P. ultimum* produces metabolites toxic to geranium and that the metabolites may be important determinants in disease development. In vitro plantlets exposed to the metabolites showed symptoms similar to infections by *Pythium* on geraniums and, more importantly, cultivar sensitivity to the metabolites was related to susceptibility to *P. ultimum* in the greenhouse.

Emerging roots of geranium plantlets grown in contact with culture filtrates of *P. ultimum* showed necrotic lesions, discoloration of the roots, and death of epidermal and cortical cells. All are symptoms commonly associated with infections by *Pythium* (7–9,26). The thin roots and poorly developed root systems observed on treated plantlets were also recorded on geraniums infected with *P. ultimum*.

Cytological modifications have been reported to occur in various hosts infected with *Pythium* spp. (3). We clearly observed the high sensitivity of root-hair cells to *P. ultimum*. Kraft et al (13) reported the early death of these cells during infection of bentgrass roots by *P. aphanidermatum*. The high sensitivity of these cells to metabolites of *P. ultimum* may explain why infections by *Pythium* usually begin by root-hair penetration (13,18). Furthermore, the invasion of the pathogen is known to usually be confined to the cortical zone of the roots (9,18), while the cellular damages caused by metabolites of *Pythium* on roots of in vitro plantlets also were more severe in this zone. Hock and Klarman (9) noted that the root vascular cylinder of pine seedlings stays immune to *Pythium debaryanum* invasion and that the previously infected roots show a thickening of the suberized endodermis. These observations are analogous to the root alterations shown in Figure 3.

Reduction of vegetative growth of geranium and other greenhouse crops is also associated with the presence of *P. ultimum* on *Pelargonium* (6,7,12,20,26). This is likely to be caused by low efficiency in nutrient uptake by invaded roots (20), which would explain why, when the in vitro plantlets became root-dependent, a growth reduction was observed. The absence of apparent symptoms on the aerial part of the plant, also characteristic of many infections by *Pythium* (7,8,26) and noted on treated geranium plantlets and greenhouse geraniums, suggests that the

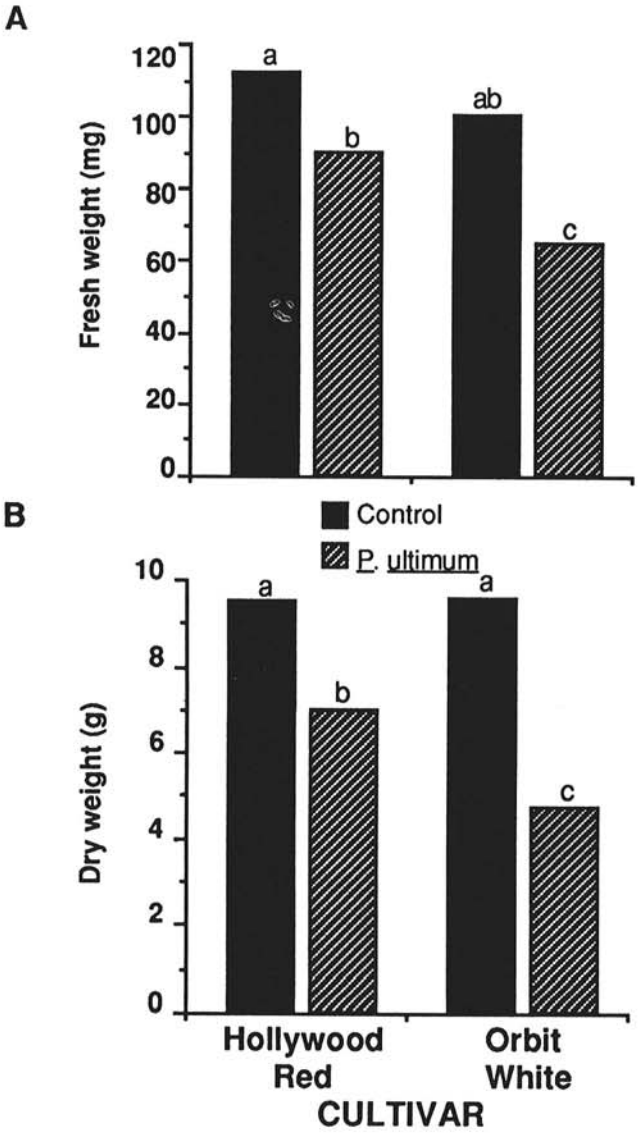

Fig. 4. A, Average fresh weight of plantlets of two *Pelargonium* × *hortorum* cultivars after 20 days of growth on a rooting medium treated with 2.5% (v/v) culture filtrates of *Pythium ultimum* or with control filtrates. **B,** Average dry weight of plants of the same cultivars grown 8 wk in a substrate infested with *P. ultimum* or in an uninfested substrate (control). Values with the same letter are not statistically different according to Student's *t* tests (LSD), $P = 0.05$.

effect of fungal metabolites is limited or confined to the root region. It is thus possible that most of the symptoms observed on geraniums and other crops as a result of infections by *Pythium* were caused by the production of toxic metabolites by the fungus. In cases in which these metabolites have weakened a plant to a high degree, *Pythium* could then invade the plant, probably through the use of hydrolytic enzymes (10), and cause its death. Supporting this hypothesis is the observation that more plants of Orbit White died during our experiments. This cultivar was more affected by the metabolites in the bioassay.

The increase in the number of roots observed on Hollywood Red plantlets, after the addition of fungal metabolites, suggests an auxinic effect of these metabolites on *Pelargonium*. Similar properties have been found on metabolites isolated from *Pythium sylvaticum* (23). The cultivar Hollywood Red was more responsive to this effect, and it may have resulted in better vegetative growth for plantlets treated with Hollywood Red than for those treated with Orbit White. This may explain why Hollywood Red tolerated infection by *P. ultimum* more than did Orbit White in both the in vitro bioassay and the greenhouse experiment.

Because other investigators have suggested the production of several toxic metabolites by *Pythium* spp. (15,16), further purification of these active compounds from culture filtrates could contribute additional information on the disease etiology. The specific biological activity of each of these compounds (applied singly and in combination) could also be measured with the use of our bioassay. This information should provide valuable insights into the biological control of *Pythium* spp.

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