Growth and Biological Control Activity of *Tilletiopsis* Species Against Powdery Mildew (*Sphaerotheca fuliginea*) on Greenhouse Cucumber

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ABSTRACT

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The ballistospore-forming yeast *Tilletiopsis* was recovered from powdery mildew-infected leaves of 22 plant species in the lower Fraser Valley of British Columbia, Canada, during 1990–1992. A semiselective medium, comprised of corn meal agar, ampicillin (100 μg/ml), dichloran (10 μg/ml), and rose bengal (20 μg/ml), was developed to enhance recovery of *Tilletiopsis* isolates from nature. Among a total of 143 isolates, four species—*T. washingtonensis, T. minor, T. pallescens,* and *T. albescens*—were recovered in decreasing frequencies. Two isolates, one of *T. pallescens* and one of *T. washingtonensis*, were evaluated for their growth response to environmental factors and efficacy in reducing powdery mildew growth and sporulation. Growth of the isolates on agar medium was similar at 15–30 C but was significantly ($P = 0.05$) reduced at 3 C; optimal growth and spore production in broth occurred at 15–25 C, and growth was nil at 30–35 C. Ballistospores germinated over the pH range of 3.8 to 7.9. Reducing the osmotic potential in liquid culture with PEG 8000 from −0.5 to −2.0 MPa resulted in decreased biomass and blastospore production along with a change in growth from blastospore production to mycelium production. Among several culture media that were compared in this study, optimal blastospore production occurred on 2.5% D-glucose, 1.0% peptone, and 0.1% yeast extract. When a blastospore suspension containing 1×10^8 cells per milliliter was applied to the surface of cucumber leaves under conditions of 85% relative humidity and 25 C, the yeast could be recovered from leaf washings over a 4- to 5-wk period. Applications of *T. pallescens* or *T. washingtonensis* to naturally infected greenhouse cucumber plants during 1991, three times at weekly intervals, reduced the mildew conidia density significantly ($P = 0.0001$) when compared to a diluted broth control. In two subsequent trials, *Tilletiopsis* treatments significantly ($P \leq 0.05$) reduced mildew sporulation and hyphal growth when compared to the control. Mildew hyphae after *Tilletiopsis* treatment were shrunken and collapsed when viewed under the scanning electron microscope, and the conidiophores had fewer spores. Growth of *Tilletiopsis* occurred on laminarin as a sole carbon source in vitro, but there was no growth on chitin. Activity of β-1,3 glucanase was detected and could be one of the potential modes of action of this biological control agent against powdery mildew.

Additional keywords: antagonism, greenhouse vegetables, hydrolytic enzymes, phylloplane.

Powdery mildew, caused by *Sphaerotheca fuliginea* (Schlectend:Fr.) Pollacci, is a prevalent disease on cucumber (*Cucumis sativus* L.) grown for fresh-market production in greenhouses in British Columbia, Canada (7). Methods for disease control currently available to commercial growers include repeated applications of elemental sulfur (2), use of sodium silicate in the hydroponic nutrient solution (24), and utilization of mildew-tolerant cultivars. However, none of these methods has provided an adequate level of disease control, and each has its limitations (24,33).

Several potential biological agents have been described for control of powdery mildews. These include the hyperparasitic fungus *Ampelomyces quisqualis*, which inhibits conidial production and cleistothecial formation (29); *Acremonium alternatum*, a pycnidium-forming hyperparasite (22); *Stephanoascus* spp. (anamorph: *Sporothrix*), an ascomycetous yeast that reduces mildew growth and sporulation (17), and *Tilletiopsis* spp., a ballistospore-forming yeast that reduces powdery mildew development on both cucumber (14,16) and barley (18). Both *Stephanoascus* and *Tilletiopsis* spp. appear to have potential as biological control agents for powdery mildew on rose and cucumber under commercial growing conditions in eastern Canada (17) and in the Netherlands (15), respectively.

Due in part to the unavailability of effective control measures for powdery mildew on cucumber in British Columbia, we evaluated the efficacy of *Tilletiopsis* spp. as a biological control agent. This ballistospore-forming yeast occurs naturally on mildew-infected leaves of a number of plant species (4,6,20) and has shown some potential in studies conducted in the Netherlands (13) and Denmark (19). In spite of this, there is very little published information on several aspects that are critical to the successful use of *Tilletiopsis* as a biological control agent, including optimal conditions for growth and inoculum production, factors influencing spore germination and survival of the antagonist, and potential mode(s) of action. Furthermore, the efficacy of *Tilletiopsis* as a biological agent under growing conditions in British Columbia has not been investigated previously.

The objectives of this study were to 1) determine the prevalence of *Tilletiopsis* spp. on mildewed leaves in nature; 2) study the environmental and nutritional factors influencing inoculum production and viability; 3) elucidate the potential mechanism of action against powdery mildew; and 4) evaluate the potential of this yeast as a biological control agent under commercial growing conditions. Preliminary results from this study have been published (31,32).

MATERIALS AND METHODS

Survey and isolation of *Tilletiopsis* spp. During April through September in each of 3 yr (1990–1992), leaf samples were collected arbitrarily from a total of 22 plant species on which powdery mildew infection was visible. The plants sampled were maple (*Acer* spp.), horse chestnut (*Aesculus hippocastanum* L.), azalea (*Rhododendron* spp.), calendula (*Calendula officinalis* L.), curcubits (*C. sativus* and *Curcubita pepo* L.), lupine (*Lupinus* spp.), apple (*Malus ala* L.), sweet clover (*Melilotus officinalis* (L.) Lam.), mint (*Mentha* spp.), plantago (*Plantago major* L.) poplar (*Populus tremuloides* Michx. Pursh.), cherry (*Prunus* spp.), rhododendron (*Rhododendron* spp.), hybrid rose (*Rosa* spp.), salmonberry (*Rubus parviflorus* Nutt. and *R. spectabilis* Pursh.), dock (*Rumex crispus* L.), elder (*Sambucus cerulea* Raf.), dandelion (*Taraxacum officinale* Wigg.), clover (*Trifolium pratense* L.), wine grape (*Vitis* spp.) and zinnia (*Zinnia elegans* Jacq.). Diseased leaves were collected from various locations in the greater Vancouver area and throughout the Fraser Valley of British Columbia. Most samples originated from gardens, parks, or recreational areas, except for cucumber leaves, which were obtained from commercial greenhouses. In addition, leaves of various plant species without visible powdery mildew infection also were sampled at various times during 1991 and 1992.

The samples were brought back to the laboratory, and isolation of epiphytic fungi, including *Tilletiopsis*, was attempted. Entire leaves or leaf sections (~5 cm^2) with powdery mildew colonies were attached to the lid of a petri dish (100 × 25 mm) with adhesive tape (sporulating surface down) and incubated at 20 C for 18–36 h. The bottom of the petri dish contained corn meal agar (CMA, Difco Laboratories, Detroit) amended with dichloran (Botran 75 WP) at 10 μg/ml a.i. and ampicillin at 100 μg/ml. After incubation, the leaf segments were removed, and the petri dishes were incubated at 20 C for an additional 7–10 days to allow fungal colonies to develop. Putative colonies of *Tilletiopsis* were subcultured onto malt extract agar (MEA, Difco). They were identified by their yeast-like appearance, the colony morphology, and the presence of long, thin, sickle-shaped yeast spores or ballistospores (4). The identity of each isolate was confirmed according to the criteria of Boekhout (4) and Nyland (26), which included the presence of delicate pseudohyphae or hyphae with one sickle-shaped spore per hypha attached by a short sterigma and formation of chlamydospores and disarticulate hyphal sections separated by dead sections with numerous septa.

Development of a semiselective medium for recovery of *Tilletiopsis*. The abundant growth of contaminating microorganisms, such as *Alternaria, Penicillium, Cladosporium,* and *Botrytis*, and various bacteria from many of the isolations attempted from naturally infected leaves during the surveys conducted in 1990 and 1991 necessitated the development of a semiselective medium. Various concentrations and combinations of tetracycline hydrochloride, chloramphenicol, ampicillin, rose bengal, and benomyl were added to CMA containing dichloran (10 μg/ml a.i.) and ampicillin (100 mg/ml a.i.); these were evaluated for enhanced recovery of *Tilletiopsis*. Growth of pure cultures of *Tilletiopsis* spp. also was evaluated on various media. In addition, recovery of the organism from artificially inoculated cucumber leaves was tested on various modified media.

Growth of *Tilletiopsis* in broth culture. Growth characteristics of two isolates of *Tilletiopsis* identified as *T. pallescens* Gokhale and *T. washingtonensis* Nyland by T. Boekhout (Centraalbureau voor Schimmelcultures, Baarn, the Netherlands), both isolated from mildewed cucumber leaves, were evaluated in the following broth media (ingredients per liter of water): 1) 2 g of peptone (Bacto, Difco) and 20 g of malt extract (Difco); 2) 10 g of peptone (Bacto) and 20 g of malt extract; 3) potato-dextrose broth (PDB; Difco); 4) 10 g of peptone (Bacto), 25 g of glucose, and 1 g of yeast extract (designated T II broth); 5) PDB (Difco) with 10 g of peptone; and 6) 25 g of glucose, 1 g of yeast extract, and 10 g of ammonium nitrate. A 50-ml volume was dispensed into a 125-ml flask, inoculated with a 4-mm-diameter plug from MEA, and the flask was placed on a rotary shaker (150 rpm) at 20 C under fluorescent lamps. The extent of mycelial growth, spore production, and development of chlamydospores was measured from two replicate flasks after 72 h of growth. The mycelium was filtered through a Buchner funnel lined with Whatman No. 540 filter paper and dried at 40 C for 72 h. To quantify spore production, a 0.2-ml sample was withdrawn from the flask at 12-h intervals for up to 72 h, and the spore density was estimated with a haemocytometer. A minimum of two replicate counts was made for each flask, and the experiment was conducted twice.

Influence of temperature and osmotic potential on growth and sporulation. To evaluate the influence of temperature on colony growth of *Tilletiopsis*, petri dishes (100 × 15 mm) containing MEA were inoculated with a 4-mm-diameter plug of either *T. pallescens* or *T. washingtonensis* taken from 14-day-old cultures and placed at 3, 15, 20, 25, 30, or 35 C. The dishes were incubated upside down to minimize lateral spread of colonies by ballistospore discharge. Colony diameter was measured from five replicate plates every four days over a 30-day period and averaged. In addition, both *Tilletiopsis* spp. were grown in T II broth at temperatures of 5–30 C, at 5 C increments, and measurements of mycelial dry weight accumulation and sporulation were made after 72 h as described above.

The influence of osmotic potential on growth and sporulation was determined using T II broth amended with varying amounts of polyethylene glycol (PEG 8000) to achieve osmotic potentials that ranged from −0.5 to −2 MPa. Nominal osmotic potentials of the amended media were calculated by osmotic coefficients (28) and formulae (25). Actual osmotic potentials of autoclaved media were measured with a SC-10 Decagon thermocouple psychrometer (Campbell Scientific Inc., Logan, UT) with a HR-33T dew point microvoltmeter (Wescor, Inc., Logan, UT). The microvolt reading was converted to −Pascal (−Pa) units (in which −1 MPa potential = −10 bars) by NaCl standard curves. Flasks were inoculated with 0.05 ml of a spore suspension from a 5-day-old broth culture. Mycelial dry weight and spore production on the osmotically adjusted media was determined after 80 h of incubation on a shaker (150 rpm) at 20 C as described above.

Influence of pH on ballistospore germination. The pH of Bacto water agar (1.5%) was adjusted with 0.05 N HCl or NaOH to achieve pH values in the range of 3.8 to 7.9. Actual pH measurements were made after the medium was autoclaved and cooled to 40 C. The medium was poured into 60 × 15 mm petri dishes and a 36-h-old culture of either *T. pallescens* or *T. washingtonensis*, which was initiated by plating a spore suspension on MEA, was suspended over the agar surface for 2 h. This resulted in the deposition of several-hundred ballistospores onto the medium. Spore germination was assessed after 24 h of incubation at 20 C from 500 randomly selected spores viewed on an inverted microscope. Spores were considered to have germinated if a germ tube was visible. For each pH treatment, five replicate dishes were included, and the experiment was conducted twice.

Carbon-source utilization and enzymatic activity. To determine whether *T. pallescens* and *T. washingtonensis* could utilize carbon sources other than glucose, carbon-free yeast nutrient medium (Difco) was supplemented per milliliter with 1 mg of colloidal chitin (prepared according to the method of Lingappa and Lockwood [21]), 1 mg of laminarin (a β-1,3 glucan polymer), or 1 mg of *N*-acetylglucosamine (Sigma Chemical Co., St. Louis). All media were inoculated with 0.06 ml of a 72-h-old spore suspension (1×10^8 spores per milliliter) of either *T. pallescens* or *T. washingtonensis* grown in T II broth. After 96 h, the contents of the flask was centrifuged at 10,000 *g* for 30 min, the supernatant was decanted, and the pellet was resuspended in distilled water and recentrifuged. The washed mycelium was separated from the supernatant, resuspended in 5 ml of water, decanted into petri dishes, and dried to constant weight at 42 C.

The activity of β-1,3-glucanase in the culture fluid after centrifugation was tested after lyophilization. The lyophilized sample from a 25-ml volume of either *T. pallescens* or *T. washingtonensis* culture was dissolved in 0.5 ml of acetate buffer (0.05 M, pH 5.0) containing 3.5 mg of laminarin and incubated for 4 h at 30 C. The enzyme activity of a *Trichoderma harzianum* isolate

was analyzed as for *Tilletiopsis* to serve as a positive control after incubation for 2 h. The glucose released from laminarin hydrolysis by both fungi was determined with an enzyme-based glucose determination kit (Sigma). A unit of enzyme activity (glucose units) was expressed as micromoles of glucose produced per milligram of crude protein per hour of incubation. Crude protein content of the lyophilized culture filtrate was determined by the method of Bradford (5) with bovine serum albumin (Sigma) as the standard. The experiment was conducted three times.

Duration of survival of *Tilletiopsis* on the leaf surface. Five-week-old cucumber plants, cv. Calypso, grown at constant 25 C and 85% relative humidity with 16 h of light/8 h of dark were used. A blastospore suspension of *T. pallescens* (10^8 spores per milliliter) from a 3-day-old T II broth culture was sprayed uniformly with a hand-held atomizer onto each of six fully expanded leaves (total volume applied per leaf = 0.2 ml). The plants were returned to the growth chamber after the leaves had dried, and the day 0 population was determined 12 h after application of *Tilletiopsis*. At weekly intervals for up to 6 wk, a single leaf was removed at random from four replicate plants, cut into sections, and placed in a 250-ml bottle containing 100 ml of 0.1% Bacto-peptone solution (Difco). The suspension was shaken at 250 rpm for 10 min, at which time serial dilutions (up to 10^{-3}) were made, and a 0.1-ml sample was plated onto the semiselective medium. Two replicate dishes were included at each sampling time and incubated at 20 C for 14 days, at which

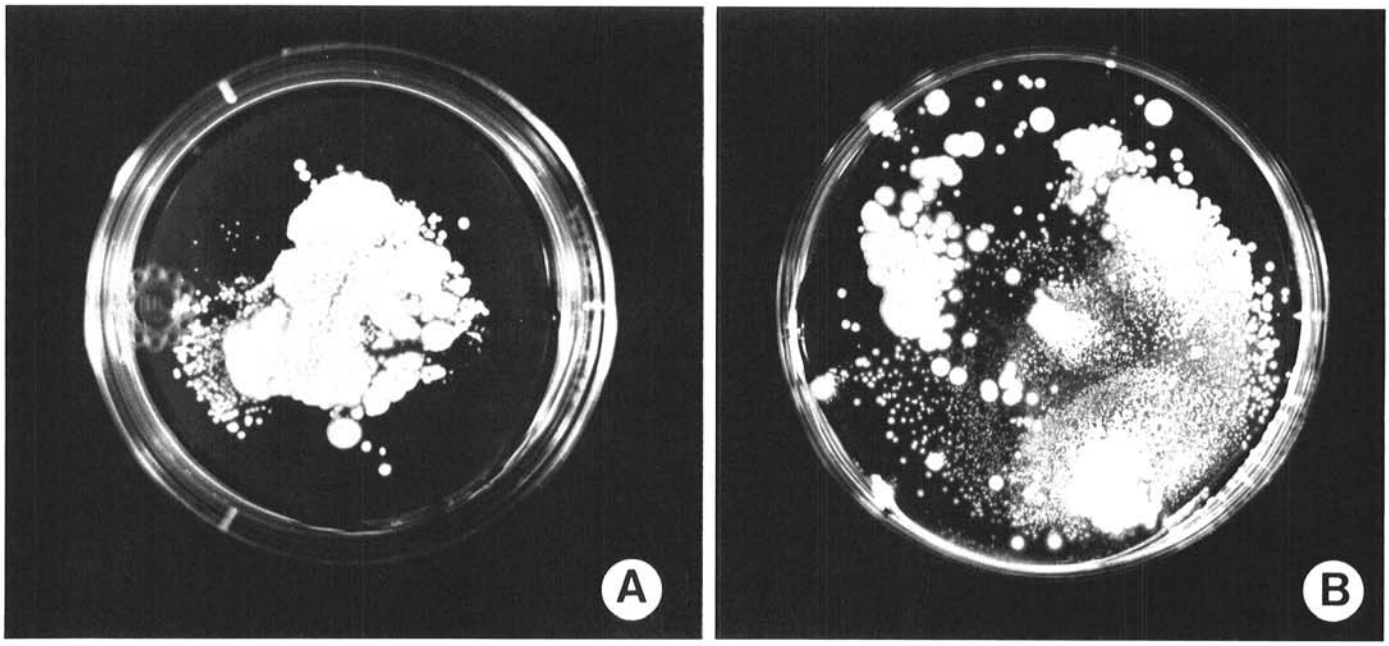

Fig. 1. Morphological characteristics of *Tilletiopsis* spp. **A,** Two-week-old colony of *T. washingtonensis* growing on malt extract agar; and **B,** *T. pallescens* showing numerous satellite colonies resulting from ejected ballistospores from the parent colony.

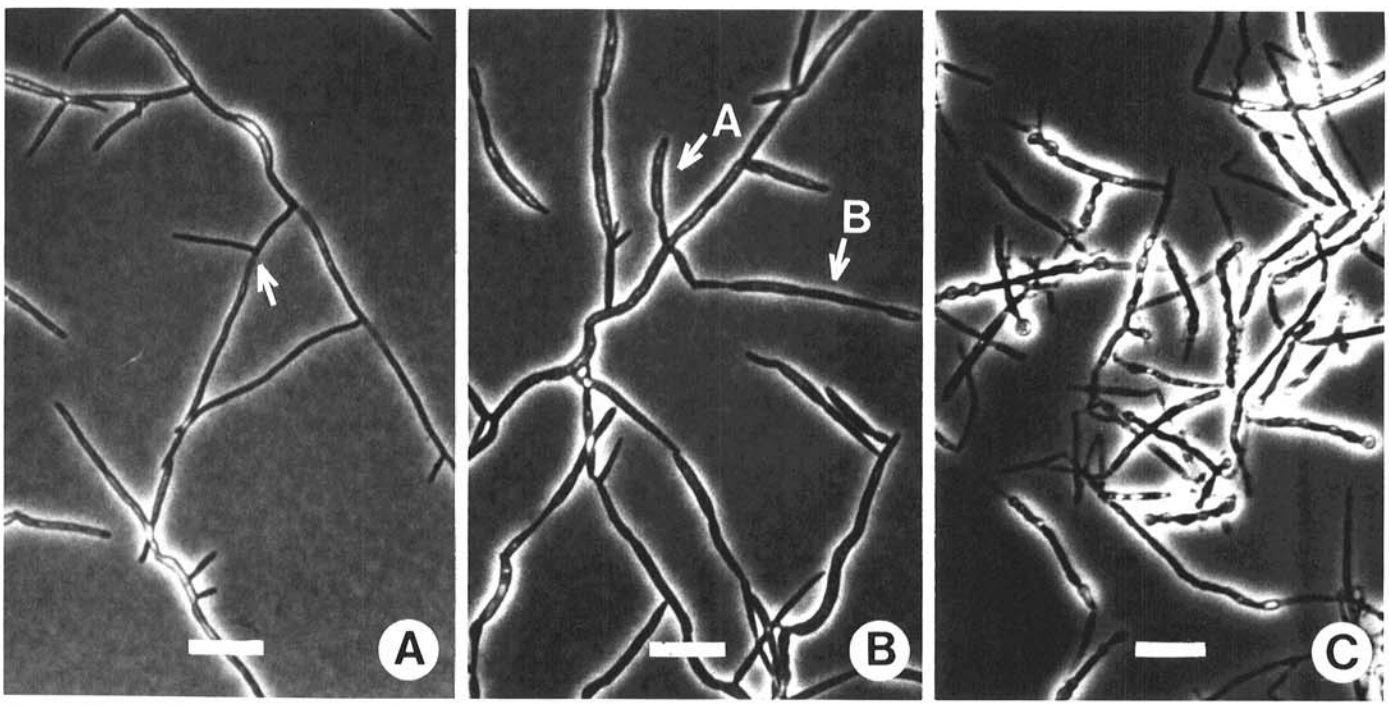

Fig. 2. Microscopic characteristics of *Tilletiopsis* spp. **A,** Disarticulate live hyphal segments with retracted cytoplasm separated by hollow hyphal sections with septa in *T. pallescens* on potato-dextrose agar (PDA) (branching at arrow); and **B,** blastospores (arrow A) and repetitive spores (arrow B) attached to sterigma of 24-h-old *T. pallescens* colony on PDA; **C,** chlamydospores of *T. washingtonensis* from T II broth (10 g of peptone [Bacto], 25 g of glucose, and 1 g of yeast extract per liter of water) after 7 days of growth. Bars = 10 μ.

time the number of *Tilletiopsis* colonies recovered was determined. The experiment was repeated twice, once with a blastospore suspension and once with chlamydospores (7-day-old culture) as the inoculum.

Evaluation of *Tilletiopsis* for biological control activity. During the growing season (May to September) during 1991 and 1992, *T. pallescens* and *T. washingtonensis* were evaluated for biological control of cucumber powdery mildew. These experimental trials were conducted twice during 1991 and once during 1992 in a commercial-size experimental greenhouse under conditions similar to those for commercial production of the crop. Cucumber seedlings, cv. Corona, were initiated in rockwool cubes containing vermiculite and subsequently transferred to 1.0-×0.2-m rockwool slabs. A hydroponic nutrient solution was provided as needed to ensure optimal growth (2). A total of 30 plants was used in each trial, with 10 plants per treatment. The plants were arranged in six rows of five plants each with 1 m of spacing between adjacent plants. The treatments were 1) control-sprayed with 0.25-strength sterile broth diluted with distilled water; 2) *T. pallescens*; and 3) *T. washingtonensis*. The inoculum of both *Tilletiopsis* spp. was composed of a spore suspension (10^8 cells per milliliter) from a 3-day-old culture grown in T II broth. The contents of the flask was filtered through a triple layer of cheesecloth to exclude mycelium. The resulting spore suspension was atomized onto the upper leaf surfaces, developing fruit, stems, and flowers to run-off (about 3.5 ml per leaf) at the initiation of the experiment. The first application was made when the most distal leaf (number 10 from the cotyledon leaf, which was number 1) was fully expanded, with leaves 3–9 used in the experiment. The *Tilletiopsis* treatment was repeated again after 7 and 14 days on the same leaves. Control plants were segregated at one end of the greenhouse, and the remaining plants were randomly assigned to either *Tilletiopsis* spp. and distributed in the rest of the greenhouse.

To quantify the effect of *Tilletiopsis* applications on powdery mildew development, clear adhesive tape (17 mm wide) was used to sample the mildew conidia density on the upper leaf surface. The tape was applied on either side of the midvein (2.5 cm away) along the entire length of the leaf. At each sample time, six subsamples were obtained that included one leaf each from six of the 10 plants in each treatment. An arbitrarily chosen mix of leaf ages and plants was selected at each sample time. The tape was subsequently attached to glass microscope slides and segmented into 7.5-cm-long pieces. The mildew conidia density was determined by counting eight fields per slide at equal distances along the length of the slide. This method provided eight slides per leaf for a total of 64 counts per leaf and 380 counts per sample. The sampling method was chosen to provide a completely random evaluation at each sample time and to minimize the influence of leaf age or application procedure on mildew assessment. Mildew spore counts per microscope field were averaged and converted to conidial density per square centimeter of leaf surface. The data were analyzed by the general linear modeling program (GLM) in the SAS statistical software package (SAS Institute Inc., Cary, NC). Comparison means between different analysis of variance (ANOVA) cells were confirmed by Bonferroni's method. The mean mildew conidial densities were log-transformed in all trials to conform to the homoscedasticity requirements of the ANOVA.

Scanning electron microscopy (SEM). Mildewed cucumber leaves, with and without *Tilletiopsis* treatment, were collected from plants in the May 1991 greenhouse trial after two applications (11 days) and processed for SEM. Tissue pieces, ~5 mm^2, were fixed in 3% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 1 h, followed by exposure to osmium tetroxide vapor for 24 h, and then rinsed three times in distilled water. The samples were dehydrated through a graded ethanol series (from 10 to

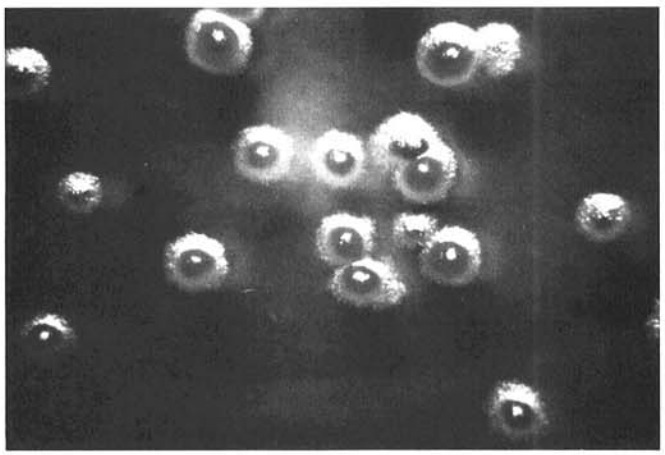

Fig. 3. Colonies of *Tilletiopsis* developing on semiselective medium (cornmeal agar with 10 μg of dichloran, 100 μg of ampicillin, and 20 μg of rose bengal per milliliter). Bar = 2 mm.

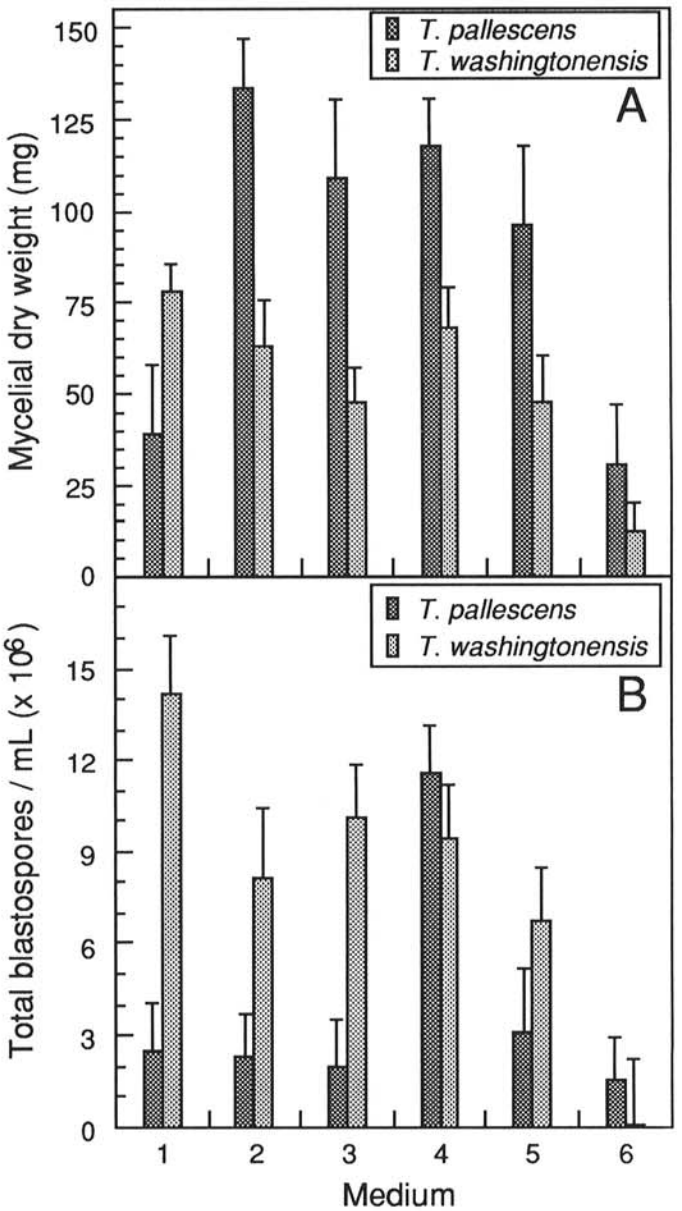

Fig. 4. Effect of various media on *Tilletiopsis* growth in broth cultures after 72 h (ingredients per liter of water): 1) 2 g of peptone (Bacto) and 20 g of malt extract; 2) 10 g of peptone (Bacto) and 20 g of malt extract; 3) potato-dextrose broth (PDB); 4) 10 g of peptone (Bacto), 25 g of glucose, and 1 g of yeast extract (designated T II broth); 5) PDB with 10 g of peptone; and 6) 25 g of glucose, 1 g of yeast extract, and 10 g of ammonium nitrate. **A,** Mycelial dry weight production; and **B,** blastospore production. Vertical bars represent standard error of the mean ($n = 8$).

100%, 30 min in each) and critical point-dried with liquid CO_2. The specimens were mounted on stubs with double-sided adhesive tape, coated with gold, and viewed in a scanning electron microscope. Photographs were taken with Kodak TMX-3045 film.

RESULTS

Survey and isolation of *Tilletiopsis* spp. A wide range of plants naturally infected with powdery mildew fungi were surveyed for the presence of *Tilletiopsis* colonies during April to September during each of 3 yr (1990–1992), including cucumber plants grown in commercial greenhouses in the Fraser Valley of British Columbia. *Tilletiopsis* isolates were obtained from all the mildewed leaves of these plant species. Numerous colonies developed on the isolation plates, and representative ones were selected and subcultured. All isolates conformed to the botanical description of the genus *Tilletiopsis* (4,26) and were compared with type cultures provided by other researchers. A total of 143 isolates comprising four species have been obtained to date. In general, colonies of *T. washingtonensis* were more numerous than *T. minor* Nyland, with putatively identified colonies of *T. pallescens* or the closely related *T. albescens* Gokhale rarely seen. All mildewed leaves that had *Tilletiopsis* present generally yielded colonies of *T. washingtonensis* (Fig. 1A) and *T. minor* (Fig. 1B). Colonies that were identified as either *T. albescens* or *T. pallescens* were only recovered from infected leaves of greenhouse cucumbers and salmonberry. Isolates of *T. washingtonensis* were the first species to be isolated during spring (April through May) and were the only colonies present after a killing frost. The other *Tilletiopsis* spp. were recovered at various times between May and September. Despite repeated attempts to obtain *Tilletiopsis* isolates from leaves without mildew or rust infections by the spore fall method and semiselective medium (e.g., dandelion [*Taraxacum officinale*], salmonberry [*Rubus spectabilis*], big leaf maple [*Acer macrophyllum*], poplar [*Populus tremuloides*], elder [*Sambucus caeruleana*], and curled dock [*Rumex crispus*]), no isolates were obtained. However, *T. minor* and *T. washingtonensis* were recovered during February to April 1993 from newly opened leaves (E. J. Urquhart, J. G. Menzies, and Z. K. Punja, *unpublished data*).

The *Tilletiopsis* isolates exhibited both yeast and mycelial growth forms, with disarticulate cells separated from the mother colony by dead hyphal sections with numerous septa (Fig. 2A). Ballistospores were produced from mycelia or blastospores and were actively ejected (Fig. 2B). Chlamydospores appeared in older (2–4 wk old) cultures on MEA; however, they developed within 4–5 days after inoculation in T II liquid medium (Fig. 2C).

Semiselective medium for recovery of *Tilletiopsis*. The very slow growth rate of *Tilletiopsis* on agar media made it difficult initially to recover the organism, because leaves were contaminated with saprophytic fungi and bacteria. The major fungi were *Alternaria, Cladosporium, Botrytis,* and *Penicillium*; various yeasts, including *Sporobolomyces*, and numerous unidentified bacteria also were present. The *Tilletiopsis* colonies required at least 14 days to grow sufficiently for subculture, attaining an average diameter of 3 mm. The saprophytic fungi often overgrew these colonies within 3–4 days. The growth of contaminating fungi was greatly reduced by the spore fall method (26) in combination with the semiselective medium developed in this study (Fig. 3). Bacterial contamination was effectively limited by adding ampicillin at 100 μg/ml, and yeast colonies rarely exceeded 5–7 mm. The fungicide dichloran (compounded as Botran) at 10 μg/ml a.i. proved to be very effective against the fungi (e.g., *Penicillium* colonies grew to only 4–7 mm in 2 wk). Cornmeal agar was chosen, because preliminary tests indicated that MEA and potato-dextrose agar (PDA) enhanced growth of the contaminants. Rose bengal (10 μg/ml) was added to reduce general fungal and bacterial growth (23); the *Tilletiopsis* colonies absorbed the dye and were selectively stained pink, which further enhanced their detection and recovery (Fig. 3).

Growth of *Tilletiopsis* in broth culture. Growth of *T. washingtonensis* was typically in a yeast-like manner on agar, and when inoculated into T II liquid medium, *T. washingtonensis* rapidly formed a blastospore suspension. Blastospore production was evident within 12 h after inoculation and reached a maximum concentration of 1.0×10^8 spores per milliliter by 72 h. In contrast, *T. pallescens* required an 18-h incubation period after inoculation of mycelium into T II medium for blastospores to develop but subsequently grew rapidly to a spore density of 1×10^8 spores per milliliter within 72 h (Fig. 2B). Viability of these blastospores was 99–100% as determined by serial dilution and plating onto PDA. The amount of mycelium produced was reduced with repeated culturing of a 72-h-old blastospore culture into new T II medium. This observation held true for many of the isolates of *T. minor, T. pallescens,* and *T. washingtonensis* that were recovered in this study. Chlamydospores produced in liquid medium could be readily used to initiate a new suspension culture, which developed with a minimum of mycelial growth.

Blastospore production by *Tilletiopsis* spp. in the liquid medium described by Hijwegen (13) was unsatisfactory because the blas-

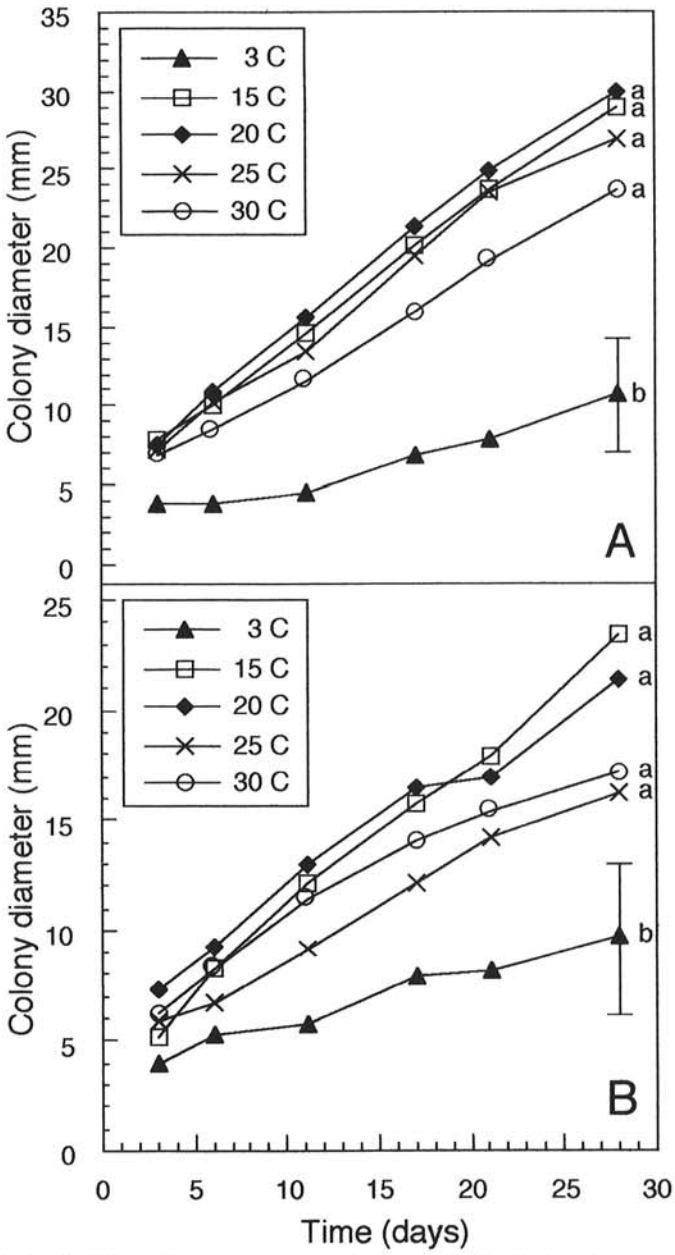

Fig. 5. Effect of temperature on radial growth of *Tilletiopsis* spp. on malt extract agar. **A,** *T. washingtonensis*; and **B,** *T. pallescens*. Means followed by the same letters at the last sampling date (28 days) do not differ significantly ($P = 0.05$ for **A** and **B**) using analysis of variance least squares mean probabilities, verified by Bonferroni's method. Vertical bars represent common standard deviation for last sampling date.

tospores were accompanied by large amounts of mycelium. When the peptone concentration was increased to 1%, blastospore production was reduced in both species, and biomass production increased (Fig. 4). PDB initially supported large quantities of mycelium in *T. pallescens*, followed by blastospore production. The addition of 1.0% peptone to PDB increased blastospore density and reduced total biomass production. Both *T. washingtonensis* and *T. pallescens* grew very poorly on medium number 6:25 g of glucose, 1 g of yeast extract, and 10 g of ammonium nitrate (Fig. 4). Medium number 4, containing 2.5% glucose, 1.0% Bacto-peptone and 0.1% yeast extract, was chosen for regular inoculum production. This medium (T II) consistently gave a high ratio of blastospores to mycelium in both species (Fig. 4).

Effect of temperature and osmotic potential (ψ_s) on growth and sporulation. Temperature had no significant ($P = 0.05$) effect on the radial expansion of *Tilletiopsis* colonies maintained at 15–30 C (Fig. 5A and B). The optimum temperature range for growth of both *T. pallescens* and *T. washingtonensis* was 15–25 C, and growth was significantly reduced at 3 C. At 35 C, all colonies were dead within four days after inoculation (E. J. Urquhart, J. G. Menzies, and Z. K. Punja, *unpublished data*). The effect of temperature on total biomass and blastospore production in broth culture is shown in Figure 6. Optimal levels of blastospores ($6–7 \times 10^7$ spores per milliliter) were obtained between 15 and 25 C. Biomass production was greatest at 25 C and negligible at 5, 10, and 30 C for both species of *Tilletiopsis* (Fig. 6). Total mycelial biomass was reduced with decreasing ψ_s from -0.5 to -2 MPa in both *Tilletiopsis* spp., although *T. pallescens* appeared to be more tolerant (Fig. 7A). In general, both biomass and blastospore production were highest in unamended T II broth (-0.5 MPa) and decreased with decreasing ψ_s. At -2 MPa, about 1.2×10^5 spores per milliliter was produced (Fig. 7B).

Effect of pH on ballistospore germination. In both *T. pallescens* and *T. washingtonensis*, germination was 100% after 24 h over the pH range tested, and new ballistospores formed except at pH 3.9, in which only mycelium or budded spores were present in *T. pallescens* and *T. washingtonensis*, respectively. After germination, *T. pallescens* produced septate mycelia from which ballistospores developed, whereas *T. washingtonensis* reproduced first by budding with pseudomycelium appearing 48 h after inoculation (Fig. 2A and B).

Duration of survival of *Tilletiopsis* on the leaf surface. The initial *Tilletiopsis*-population density on the leaf was 2.7×10^4

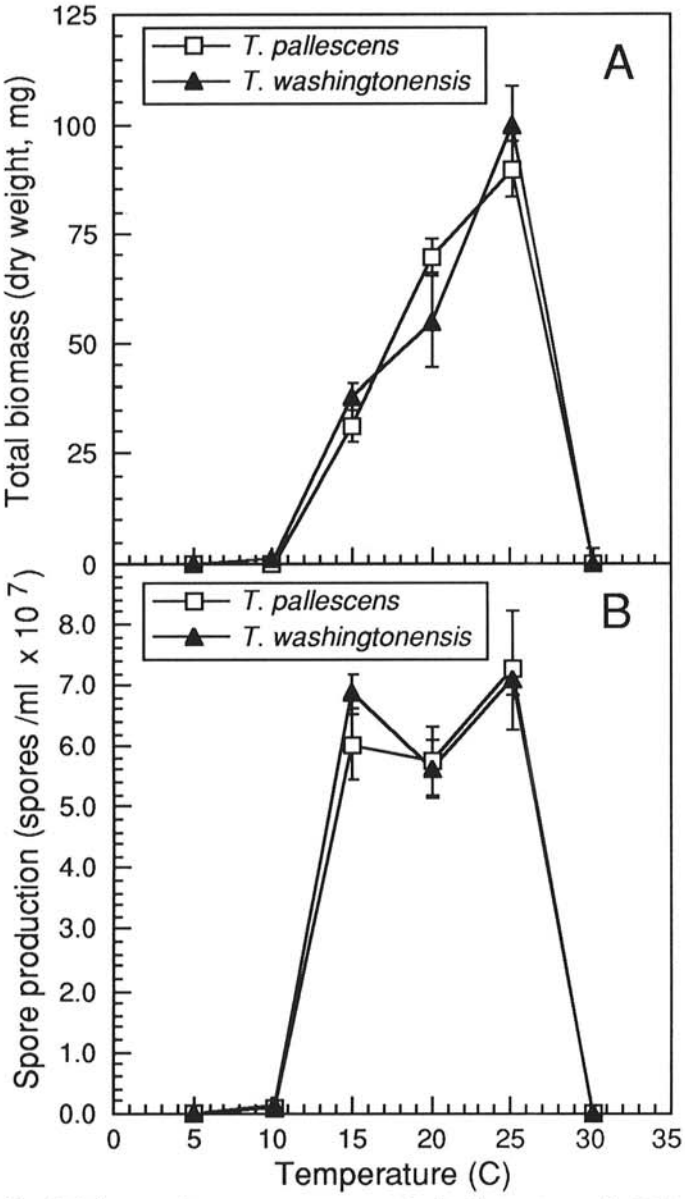

Fig. 6. Influence of temperature on growth of *Tilletiopsis* spp. in T II broth (10 g of peptone [Bacto], 25 g of glucose, and 1 g of yeast extract) cultures. **A,** Biomass production; and **B,** blastospore production. Vertical bars represent standard error of the mean.

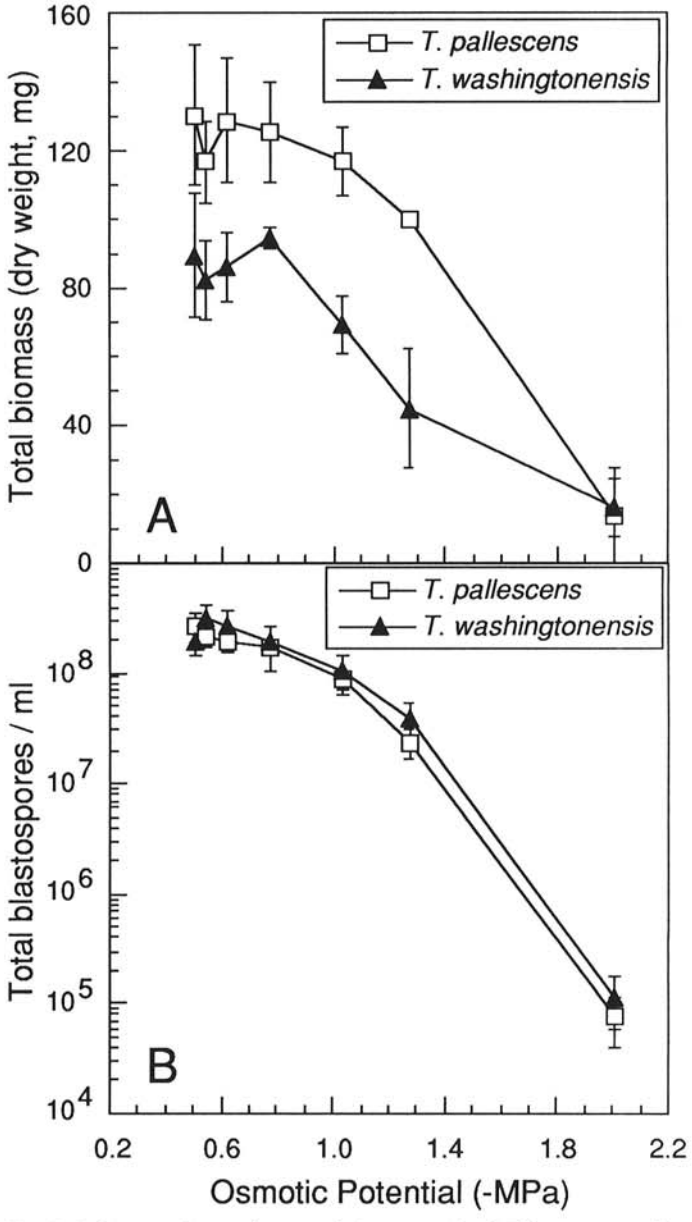

Fig. 7. Influence of osmotic potential on growth of *Tilletiopsis* spp. in T II broth (10 g of peptone [Bacto], 25 g of glucose, and 1 g of yeast extract per liter of water) culture. **A,** Biomass production; and **B,** blastospore production. Vertical bars represent standard error of the mean.

cfu at the start of the first survival trial and declined by approximately 1 log magnitude in each successive week (Fig. 8). After five weeks, 1 cfu was detected on the agar medium. The results from all three trials were similar.

Evaluation of *Tilletiopsis* for biological control activity. Naturally occurring powdery mildew infection was greater than 75% of the leaf area on leaves 8–9 and 100% on all lower leaves in the first experimental trial (initiated during May 1991). In the second experimental trial (initiated during August 1991), mildew infection was 100% on leaves 1–2 and about 15% on leaves 3–9. In the third experimental trial (initiated during March 1992), mildew infection covered 100% of the upper surface of leaves 2–9. Average mildew conidia density was reduced by one application of *T. pallescens*, *T. washingtonensis*, or the broth control (Fig. 9A) in the May 1991 trial. However, the *Tilletiopsis* treatments were significantly different ($P = 0.0001$) from the broth control by day 8. At this time, the mildew conidia density was reduced by 3.8×10^4 and $4.7. \times 10^4$ conidia per square centimeter in the *T. pallescens* and *T. washingtonensis* treatments, respectively, when compared to the broth control. The mean conidial density was similar in the *T. pallescens* and *T. washingtonensis* treatments after the second application, whereas in the broth control the average conidia density was significantly higher ($P = 0.05$) at all times after the second application.

In the August 1991 trial, the *T. pallescens* and *T. washingtonensis* treatments significantly reduced ($P = 0.001$) mildew conidia density when compared to the broth treatment at 11 days (Fig. 9B). The *T. pallescens* treatment was significantly better ($P = 0.002$) than the *T. washingtonensis* treatment. Treatments with *T. pallescens* resulted in a lower mildew conidia density than did the broth treatment ($P = 0.001$) on days 8, 11, and 15, whereas *T. washingtonensis* differed from the broth treatment ($P = 0.001$) only on days 11 and 15.

The results from the April 1992 trial (Fig. 9C) were less definitive than the two previous trials, due in part to the much higher initial disease level. Treatments with *T. pallescens* resulted in lower mildew conidia density than did the control treatment ($P = 0.01$) on days 4, 8, and 15, whereas *T. washingtonensis* differed from the control treatment ($P = 0.01$) on days 4, 11, and 15.

The effect of the *Tilletiopsis* treatments was markedly apparent on the leaves (Fig. 10). At the beginning of the May 1991 experiment, all leaves were heavily infected (Fig. 10A). After one

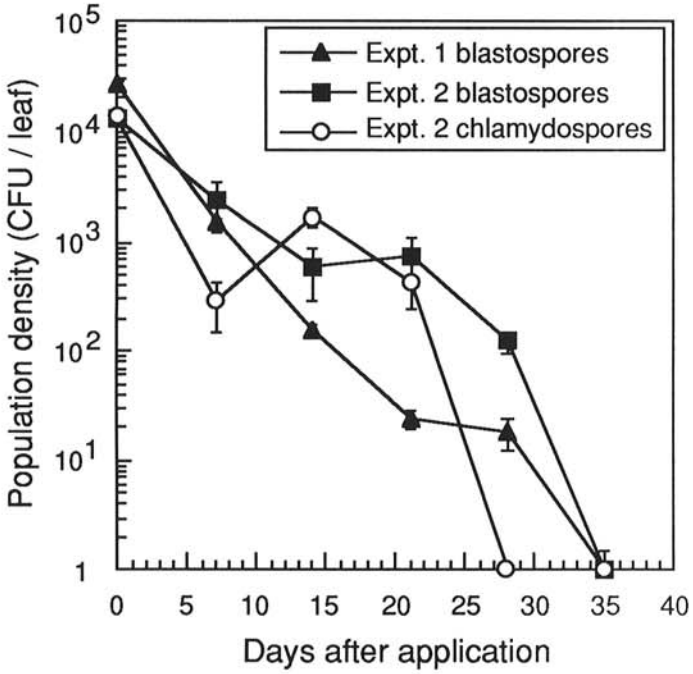

Fig. 8. Duration of survival of *Tilletiopsis pallescens* on the surface of cucumber cv. Calypso leaves maintained at 25 C and 85% relative humidity. Experiments were conducted with either blastospores or chlamydospores as inoculum. Vertical bars represent standard error of the mean.

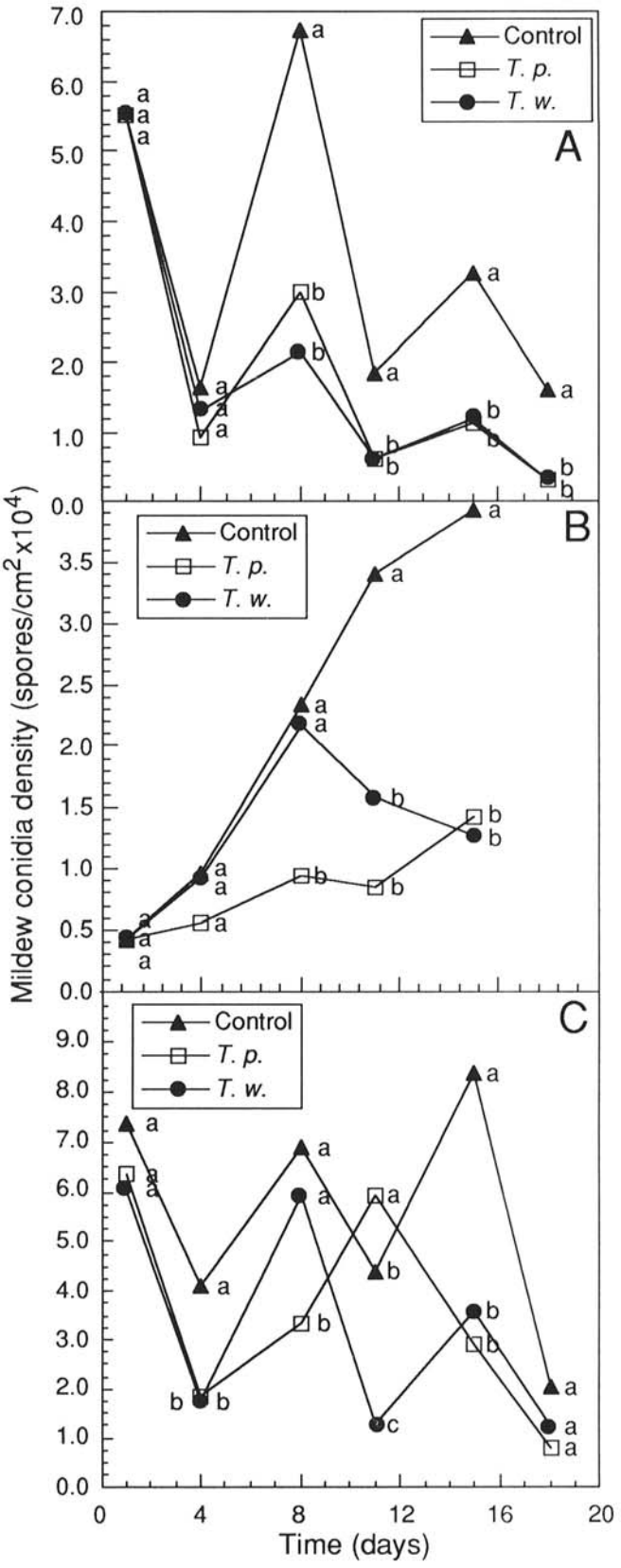

Fig. 9. Biological control activity of *Tilletiopsis pallescens* (*T. p.*) and *T. washingtonensis* (*T. w.*) against powdery mildew (*Sphaerotheca fuliginea*) on greenhouse cucumbers under commercial growing conditions. **A,** May 1991 trial; **B,** August 1991 trial; and **C,** April 1992 trial. Applications were made on days 1, 8, and 15. Means followed by the same letter do not differ significantly ($P = 0.01$ for **A** and **B**; $P = 0.05$ for **C**) for mildew conidia density at the same sampling date and represent analysis of variance least squares mean probabilities verified by Bonferroni's method.

application of the *Tilletiopsis* spore suspension, a clearing of the interveinal areas was apparent on the leaves (data not shown). Much larger areas of clearing were apparent after two *Tilletiopsis* sprays (Fig. 10B), and the leaves were almost clear of visibly mildewed areas after three applications of the *Tilletiopsis* spore suspension (Fig. 10C). A direct comparison of the effect of sterile broth and *Tilletiopsis* treatments is shown in Figure 10D.

SEM. SEM of cucumber leaves treated with *T. pallescens* or sterile broth showed that *Tilletiopsis* treatment appeared to affect the mildew hyphal integrity, resulting in collapse (Fig. 11A). The hyphae on leaves treated with broth were turgid, with well-formed conidiophores (Fig. 11B). Interspersed around the *Tilletiopsis*-treated mildew hyphae were numerous fine hyphae resembling those of *Tilletiopsis* and production of large masses of ballistospores (Fig. 11C).

Carbon-source utilization and enzymatic activity. Both *T. pallescens* and *T. washingtonensis* utilized glucose and laminarin, but not colloidal chitin or *N*-acetylglucosamine, as sole carbon sources in carbon-free yeast nutrient medium. Utilization of laminarin, but not chitin or *N*-acetylglucosamine, provided indirect evidence of the production of β-1,3-glucanase and the absence of chitinase. Analysis of lyophilized culture filtrates of *T. pallescens* and *T. washingtonensis* grown with laminarin as the sole carbon source yielded β-1,3-glucanase values of 4.8 and 3.7 μmol of glucose per milligram of crude protein per hour, respectively. By comparison, the β-1,3-glucanase values produced by

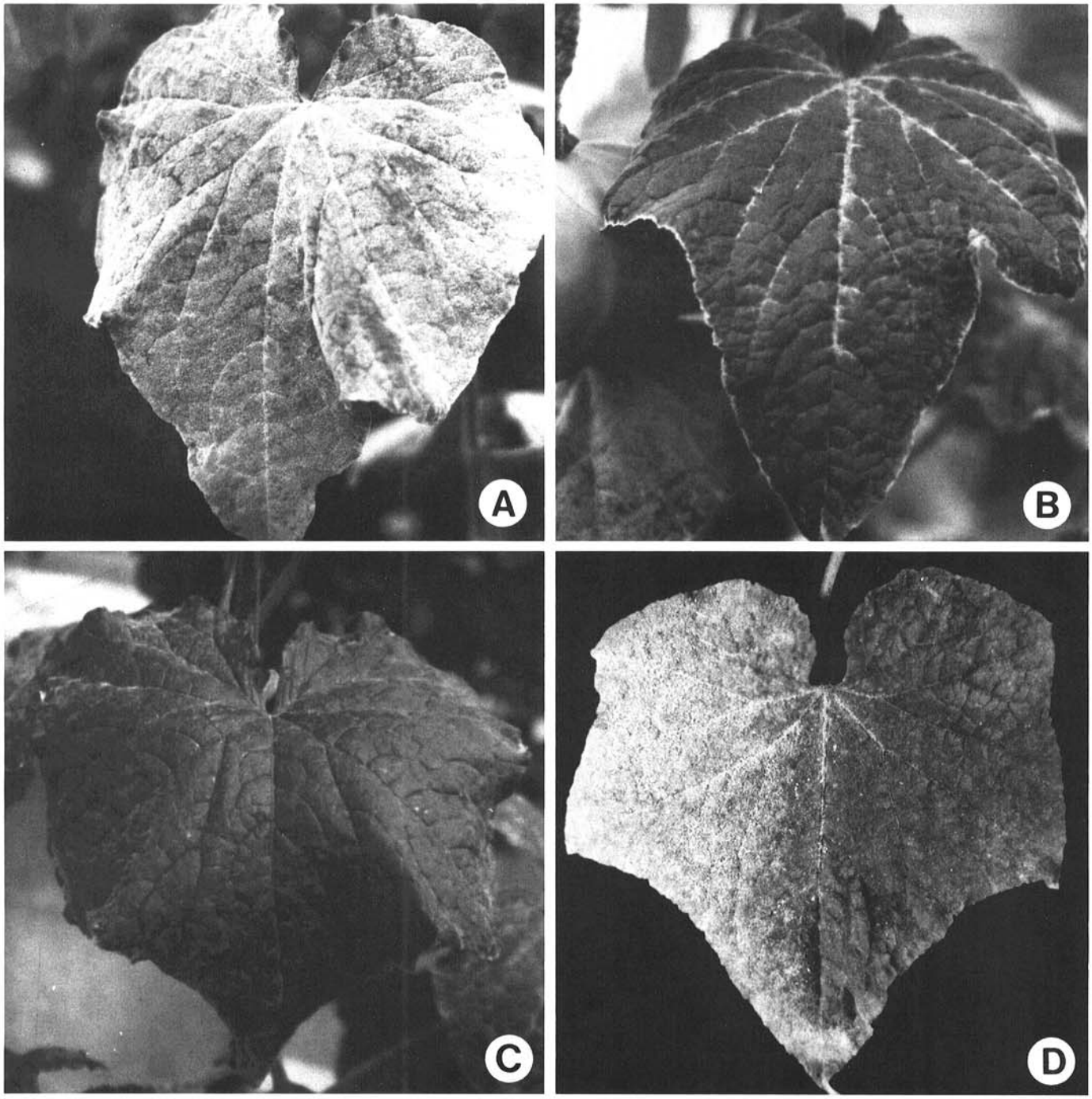

Fig. 10. Biological control activity of *Tilletiopsis pallescens* against powdery mildew (*Sphaerotheca fuliginea*) on greenhouse cucumbers. **A,** Mildew infection on untreated plants at the initiation of the May 1991 trial. **B,** Areas of clearing in the interveinal areas after two applications of a *Tilletiopsis* spore suspension. **C,** Larger areas of clearing after three applications. **D,** Comparison of mildew growth on a leaf sprayed with sterile broth (left) or *Tilletiopsis* (right).

Trichoderma harzianum grown with laminarin as the sole carbon source was 6.1 μmol of glucose per milligram of crude protein per hour.

DISCUSSION

The occurrence of species of *Tilletiopsis* on 22 species of plants, both wild and cultivated, was consistently associated with powdery mildew infections, because *Tilletiopsis* was not isolated from disease-free leaves. This agrees with Last's (20) findings, which showed that *Tilletiopsis* was a common phylloplane species in Britain on plants with powdery mildew infections but was generally not recovered from uninfected leaves. Nyland (26) also recovered numerous isolates of *Tilletiopsis* in eastern Washington from various leaves during the summer and fall seasons. The more frequent recovery of *Tilletiopsis* spp. from mildew-infected leaves suggests that some association between the two organisms exists in nature, the significance of which is undetermined.

Isolates of *T. washingtonensis* that exhibited a yeast morphology (budding blastospores with pseudomycelium) were the dominant species recovered on isolation plates in this study, and this species could be isolated over a broader time period (early in the spring and late in the fall) than any of the other species. By comparison, *Tilletiopsis* isolates with a fungal morphology (true septate mycelium), of which *T. minor* was the second most frequently isolated, were less numerous and were recovered over a shorter time period. Colonies corresponding to *T. pallescens* or *T. albescens* were rarely found, with a total of five cultures out of 143 identified in this study. The predominance of *T. washingtonensis* over *T. pallescens* cannot be explained by temperature preferences, because both had very similar growth in broth and agar in response to temperature.

The slow growth rate of *Tilletiopsis* on unamended and commonly used agar media (CMA, MEA, and PDA) presented a problem during isolation, because various saprophytic fungi, e.g., *Penicillium, Botrytis,* and *Cladosporium*, rapidly overgrew the *Tilletiopsis* colonies. The problem was overcome by combining spore-fall inoculation of plates by actively sporulating *Tilletiopsis* colonies on the leaf surface, employed by Nyland (26), with inhibition of contaminating organisms by ampicillin, dichloran, and rose bengal. Dichloran did not prevent spore germination of the contaminant fungi but effectively suppressed the rapid expansion of colonies while allowing growth of *Tilletiopsis* colonies to occur. Rapid uptake of rose bengal stained *Tilletiopsis* colonies a bright pink, whereas other contaminant yeasts and fungi did not absorb the dye as rapidly. This represents the first description of a semiselective medium to enhance the recovery of *Tilletiopsis* from nature and will be useful in monitoring population density over time.

One of the objectives of this research was to develop procedures to increase *Tilletiopsis* inoculum for use in greenhouse biological control experiments. Growth in liquid medium was superior for this purpose because growth on solid agar media was slow. Several media, including one described by Hijwegen (13), were evaluated for their ability to enhance blastospore production. A medium designated as T II (2.5% D-glucose, 1.0% Bacto-peptone and 0.1% yeast extract) was superior for blastospore production and growth and yielded the least amount of mycelium for both *T. pallescens* and *T. washingtonensis*. Routinely, 1×10^8 spores per milliliter could be obtained within 72 h after inoculation at 20 C with either species. Chlamydospores were produced from the blastospores within 6 days. Malt agar-based media gave good spore production, but large amounts of diffuse mycelium were present, whereas PDB-based media enhanced both diffuse mycelium and mycelial masses. Although Boekhout (4) reported that *Tilletiopsis* spp. utilize nitrate or nitrite as the sole nitrogen source, both *T. pallescens* and *T. washingtonensis* grew very poorly on the ammonium nitrate/D-glucose medium tested and produced the least biomass and spores among all of the media tested.

Both *Tilletiopsis* spp. grew well over a wide temperature range (15–30 C) but not at 3 or 35 C. This temperature range overlaps the temperatures at which cucumber powdery mildew conidia can germinate, i.e., 22–31 C (28 C optimum) (12). Temperatures within the range of 15–30 C resulted in similar growth and spore production in both *T. pallescens* or *T. washingtonensis*, but growth was significantly reduced ($P = 0.001$) at 3 C. Gokhale (9) reported that several *Tilletiopsis* spp., including *T. pallescens* and *T. washingtonensis*, exhibited maximum growth in liquid culture between 23 and 25 C, with little growth occurring at 3 C or above 30 C. In commercial greenhouses, temperatures can range between 15 and 40 C during sunny weather. Therefore, to ensure optimal survival and growth of *Tilletiopsis*, applications would have to be made during the early morning or late evening hours. Germination of ballistospores on pH-adjusted agar was similar between pH 3.8 and 7.9, with 100% germination recorded after 24 h. Ballistospores of *T. pallescens* germinated to produce mycelium, whereas in *T. washingtonensis* they formed budding yeast cells. Gokhale (9) previously reported that growth of *Tilletiopsis* was optimal at neutral pH in broth cultures.

We examined the effect of decreasing osmotic potential (ψ) on *T. pallescens* and *T. washingtonensis* growth and sporulation. Decreasing ψ had a negative effect on biomass and blastospore production over the range tested (−0.5 to −2 MPa) in both species. *T. washingtonensis* was more sensitive to decreasing ψ than was

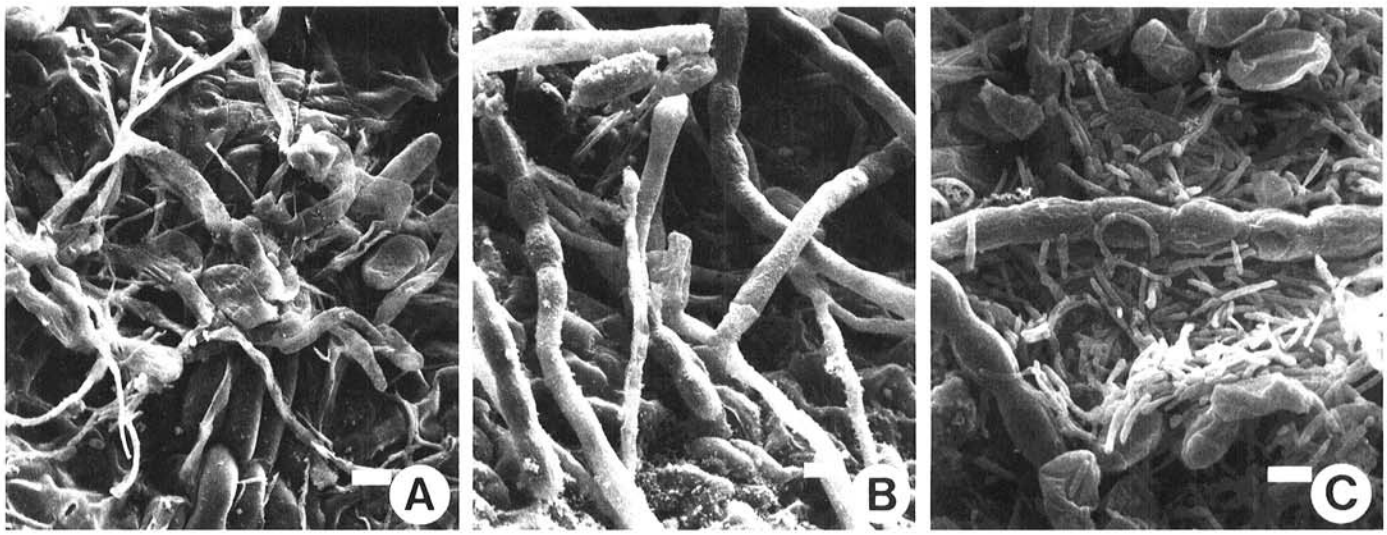

Fig. 11. Scanning electron micrographs of powdery mildew hyphae and conidia 48 h after one application of either *Tilletiopsis pallescens* or control broth. **A,** *Tilletiopsis*-treated mildew hyphae, showing collapse and loss of structural integrity; **B,** mildew hyphae in the control are turgid with well-developed conidiophores; and **C,** *Tilletiopsis* spores on leaf surface adjacent to mildew hyphae. Bars = 10 μ.

T. pallescens, as indicated by a more rapid decrease in blastospore production. Microscopic examination indicated that growth of mycelium was enhanced with decreasing ψ at the expense of blastospore production and was the only form of growth apparent at −2 MPa for both species. Based on these results, *Tilletiopsis* would likely grow as mycelium in nature unless the relative humidity was near 100%. For continued growth of the organism, relative humidity close to saturation would likely also be required. In previous studies, Hajlaoui and Bélanger (10) and Hijwegen (14) reported the high humidity requirements for optimal growth of *Tilletiopsis*.

The recovery of *T. pallescens* after application to the leaf surface declined rapidly over time at 85% relative humidity and 25 C. After 1 wk, the population had declined by 1 log unit, but *Tilletiopsis* could be recovered up to 5 wk after application. The survival capability of *Tilletiopsis* on mildewed cucumber leaves was not tested and could be higher. Different formulations of the inoculum also could reduce moisture stress, such as 1% mineral oil (27), milk (14), or spore/emulsified oil mixture (1), and warrant further study.

Three *Tilletiopsis* applications significantly reduced sporulation of *Sphaerotheca fuliginea* on heavily infected cucumber plants in this study. These results are dramatic considering that the initial disease levels were very high and the severely infected untreated plants were maintained in the same greenhouse at a distance of 0.5–0.75 m from the treated plants, allowing for continual reinoculation. The level of biological control achieved was increased with increasing numbers of *Tilletiopsis* applications. Hijwegen (13) reported in growth-chamber studies that *T. minor* greatly reduced conidiophore density and prevented regrowth of conidiophores for up to 2 wk. We have further shown that *Tilletiopsis* applications significantly reduced mildew sporulation when applied to heavily infected plants and that mildew resporulation was slower on treated plants compared to the control plants. These observations are indicative of an eradicant action of *Tilletiopsis* against powdery mildew. Protectant activity by *T. pallescens* against *Sphaerotheca fuliginea* infection was previously noted by Hoch and Provvidenti (16) on detached cucumber leaves maintained at nearly 100% humidity in petri dishes. Klecan et al (18) described a high degree of antagonism by an isolate of *T. pallescens* to *Erysiphe graminis* f. sp. *hordei* after one application to in vitro-grown barley plants. The higher relative humidity in that study probably enhanced antagonistic activity by *Tilletiopsis*. Similarly, high relative humidity was required for the mildew hyperparasite *Ampelomyces quisqualis* (27) and for antagonism by *T. washingtonensis* against rose powdery mildew (10).

The hyphae and conidia of *Sphaerotheca fuliginea* appeared collapsed when viewed under SEM after *Tilletiopsis* treatment compared with the control treatment, in which the mycelium was turgid and fully developed conidiophores were present. Mycelia of narrow diameter interspersed with mildew hyphae were likely those of *Tilletiopsis* and appeared identical to previously published micrographs of *T. pallescens* (18). The occurrence of collapsed mycelium has been observed after *T. pallescens* (formerly *Tilletiopsis* spp.) application against *Sphaerotheca fuliginea* (16). Collapse of rose mildew conidiophores after *T. washingtonensis* application also occurred within 24 h at high relative humidity (10). Knudson and Skou (19) reported that mildew mycelium was collapsed, "with a worm-eaten appearance" after treatment with *T. albescens*. In our study, ruptures in the mycelium were not seen.

During the evaluation of our *Tilletiopsis* isolates, we did not observe any broad-spectrum activity against other fungi, such as would be expected if antibiotics were produced. Other studies also have not reported any evidence of antibiotic production by *Tilletiopsis* spp. (18,30). In contrast, Hajlaoui et al (11) suggested that antibiotic production could be implicated in the activity of *Sporothrix flocculosa* against *Sphaerotheca pannosa* var. *rosae*, and no chitinase activity was detected in the interaction. However, the role of the other lytic enzymes, such as β-1,3 glucanase, was not considered. Hoch and Provvidenti (16) also implicated antibiotic action to explain hyphal collapse. We studied carbon utilization to provide preliminary insight into the action of *Tilletiopsis* against powdery mildew. Chitin and its component sugar, *N*-acetylglucosamine, were not utilized by *Tilletiopsis*, which agrees with previous reports (11,14). Laminarin (β-1,3 glucan polymer) was utilized by *Tilletiopsis*, providing indirect evidence of β-1,3 glucanase activity that was subsequently confirmed in our enzymatic assays. This enzyme has been correlated with biological control activity of various organisms, including *Trichoderma* (8) and also may be involved in the activity of *Tilletiopsis* against powdery mildew fungi. Electron microscopic studies using immunogold labeling of β-1,3 glucanase (3) should provide evidence for this proposed mode of action.

The results from this research demonstrate that *Tilletiopsis* applications greatly reduce production of conidia by *Sphaerotheca fuliginea* from very high initial levels and that this yeast is a promising candidate for biological control of cucumber powdery mildew on greenhouse cucumbers and possibly for other mildew diseases. Further research will develop methods for protectant formulations to prolong survival and to elucidate the potential spectrum of foliar pathogens that may be affected by *Tilletiopsis* spp.

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