ELUCIDATION OF THE ROLE PLAYED BY FUNGUS GNATS (DIPTERA: SCIARIDAE) IN THE TRANSMISSION OF *PYTHIUM* ROOT ROT DISEASES OF FLORICULTURE CROPS

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ELUCIDATION OF THE ROLE PLAYED BY FUNGUS GNATS (DIPTERA: SCIARIDAE) IN THE TRANSMISSION OF *PYTHIUM* ROOT ROT DISEASES OF FLORICULTURE CROPS

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Fungus gnats of the genus *Bradysia* (Diptera: Sciaridae) are ubiquitous pests in greenhouse crop-production systems. Because of their association with decaying vegetation, fungus gnats are commonly found to coexist with diseased plants, and their role as vectors of plant pathogens has been questioned for decades. Studies have demonstrated that adult fungus gnats can acquire and mechanically transmit aerial spores of various fungal plant pathogens. However, a significant role in the transmission of pathogens that do not generate large numbers of readily transmissible propagules has not been demonstrated. The ultimate objective of this dissertation was to elucidate the role played by fungus gnats, *Bradysia impatiens* (Johannsen), in transmission of *Pythium* spp. to floriculture crops. Fungus gnat adults strongly preferred to lay their eggs on plant material infected/infested with a wide array of microorganisms, including *Pythium*, *Thielaviopsis*, *Trichoderma*, *Beauveria*, and *Xanthomonas*. Although fungus gnats were highly attracted to microbial activity, adults are unlikely crop-to-crop or greenhouse-to-greenhouse vectors of *Pythium* root rot pathogens. Studies revealed that adult fungus gnats do not pick up or transmit infectious *Pythium* propagules from diseased to healthy plants. Furthermore, adult gnats do not likely carry *Pythium* internally as they are generally described as aphagous, and experiments revealed that *Pythium* ingested by fungus gnat larvae is not carried

beyond the pupal stage. Larval fungus gnats are capable of vectoring some species and strains of *Pythium* in the laboratory, although this slow-moving life stage is unlikely to account for significant transmission of *Pythium* spp. in the greenhouse setting. Feeding damage by fungus gnat larvae induced resistance to *Pythium* infection, significantly reducing seedling mortality compared with undamaged controls. The findings from these studies enhance our understanding of the association between fungus gnats and *Pythium* in greenhouse floriculture.

BIOGRAPHICAL SKETCH

Sarah E. Braun was born on May 19, 1984 in Hartford, Connecticut. She returned to Hartford in 2002 and received her B.S. with honors in Biology from Trinity College in 2006. As a Ph.D. student at Cornell, she studied the role played by fungus gnats, *Bradysia impatiens*, in the transmission of root rot disease of floriculture crops. She received grants from the National Science Foundation, the Woman's National Farm and Garden Association, and the Horticulture and Entomology Departments at Cornell. She was recognized for her scholarship and scientific achievements with the President's Prize at an Entomological Society of America meeting, membership in Sigma Xi, the George G. Gyrisco Student Award in Entomology, and a Sage Fellowship from Cornell.

Dedication

To Suzette, who always approached everyone with positive energy

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CHAPTER 1: INTRODUCTION

Fungus gnats of the genus *Bradysia* (Diptera: Sciaridae) are ubiquitous pests in greenhouse crop-production systems. Root and stem feeding and tunneling activities of *Bradysia* larvae can cause considerable direct damage to plants, especially to cuttings and seedlings. Additionally, however, fungus gnats are commonly found in association with diseased plants and decaying vegetation, and the potential of these pests to vector plant pathogens and/or predispose plants to infection by plant pathogens has been questioned for decades. The ultimate objective of this dissertation was to answer the question: what if any role does the fungus gnat *Bradysia impatiens* (Johannsen), play in *Pythium* infection of greenhouse floriculture crops.

Fungus Gnats

The dipteran family Sciaridae is comprised of small flies known as dark-winged fungus gnats. This taxonomic family has a worldwide distribution with approximately 150 described species in North America (29, 60). Fungus gnat species inhabit a variety of locations, including moist shady areas within woodlands, rodent's burrows, caves, bird's nests, greenhouses, mushroom cellars, and field crops. A review of the scientific literature on the biology of sciarids indicates that members of the genus *Bradysia* are especially abundant in greenhouse plant production (29, 60). The most common species of *Bradysia* in North American greenhouse crops include *B. impatiens* and *B. coprophila* (Linter) (29). *Bradysia* species are identified by the following morphological characters: "maxillary palpi three-segmented, usually with sensory pit, protibiae with preapical, ventral, unilateral comb separated from general tibial vestiture by triangular bare area, metatibiae with two subequal spurs, and posterior wing veins bare" (60).

Bradysia adults are weak flyers. Females average 3 mm and males 2.5 mm in length. In the greenhouse these insects are most typically found between the soil surface and lower foliage of greenhouse crops (29, 42, 66). Females typically emerge one day after the males, and the preoviposition period is less than 24 h (66). *Bradysia impatiens* females emit a sex pheromone that initiates wing fanning and upwind flight by males prior to mating (1). According to Kennedy (41), *B. impatiens* females lay an average of 156 eggs at 25°C; Wilkinson and Daugherty (66) reported a much lower fecundity of 75 eggs/female at 24°C. Adults live approximately 3 days; males typically live longer than females, which die shortly after oviposition (42, 60).

The immature stages of *Bradysia* spp. include the egg, four larval instars, and pupa. Fungus gnat larvae possess distinctive black head capsules on translucent or white bodies. In the greenhouse, the larvae are cryptic inhabitants of moist substrates such as soil, potting mixes, and decaying vegetation (66). Steffan (61) reported an average developmental time from egg to adult of 16.3 days at 20°C for insects reared on chopped sterilized straw and brewer's yeast (*Saccharomyces cerevisiae* Meyen ex E.C. Hansen), Wilkinson and Daugherty (66) reported 21.6 days at 24°C for insects reared on finely ground soybeans mixed with distilled water, and Kennedy (41) reported 15.6 days at 25°C with insects reared on brewer's yeast. The variation in developmental times reported may be due to genetic variation, diet quality, temperature, photoperiod, and/or experimental method.

There are few published accounts of feeding by adult fungus gnats and the reports are contradictory; Mercier (47) and Hungerford (34) reported feeding on nectar and a sodium arsenate/molasses solution, respectively, and Steffan (60) described them as feeding "primarily on organic ooze." However, Kennedy (41) described them as being aphagous, and attempts by Jarvis *et al*. (37) to induce fungus gnats to ingest *Pythium* oospores suspended in water with or

without 1% glucose were unsuccessful. Larvae of most sciarids feed on fungi and other microorganisms in soil or decomposing wood, including various Ascomycetes, Basidiomycetes, and Myxomycetes (41). However, the larval diets of many species consist largely of decaying plant materials or animal excrement (29, 41, 60). As previously related, larvae of several species also consume root, stem, and sometimes leaf tissue of healthy vascular plants (66). Under certain conditions, larvae may be cannibalistic (60).

Studies by Kennedy (41) indicated that *B. impatiens* larvae developed more rapidly and exhibited greater survivorship when reared on fungal diets of *Alternaria tenuis* Nees or brewer's yeast (*S. cerevisiae*) than on non-fungal diets of lima bean agar, potato dextrose agar, or agar alone. Also, in experiments with potted plants, Kennedy (42) observed *B. impatiens* larval survival to be reduced when fungal abundance (brewer's yeast) was low which, subsequently, resulted in reduced root damage. High mortality of first and second instars in pots with plants but no fungus indicated that a fungal food source is important for these age classes. In the presence of fungus, increases in fungus gnat density from 10 to 350 larvae/plant did not significantly affect larval survival and development. Olson *et al*. (51) similarly demonstrated substantially greater survival of fungus gnats in sterile potting mixes (peat moss and coir) that were supplemented versus not supplemented with yeast.

The aforementioned results are consistent with past studies indicating that sciarid fungus gnats depend upon a variety of microorganisms, including yeasts, as complete or supplementary food sources (8, 15). However, these insects are not equally attracted to all fungi as potential food sources or oviposition sites. As reported by Anas and Reeleder (5, 6), larvae of *Bradysia coprophila* Lintner preferred to feed on the sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary compared to the sclerotia of *Sclerotinia minor* Jagger and *Sclerotium cepivorum* Berk.

Furthermore, when newly hatched larvae were raised on cultures of different fungi (*Botrytis porri* Buckw., *Botrytis cinerea* Pers., *Rhizoctonia solani* Kühn, *Trichoderma viride* Pers., *S. minor*, and *S. sclerotiorum*), the mycelium and sclerotia of *S. sclerotiorum* supported most rapid and successful larval development. Larvae failed to complete development when fed cultures of the fungal antagonist *T. viride*. When selected plants were inoculated with the above plant pathogens, diseased plants supported larval development. No larvae survived on noninoculated plants (5, 6). The finding of poor survivorship among larvae fed *T. viride* is in accord with observations by Cloyd *et al*. (13) that *Bradysia* sp. nr *coprophila* adults were not attracted to a related species of *Trichoderma* (*T. harzianum* Rifai) in laboratory choice experiments.

Pythium

Species of the genus *Pythium* have a worldwide distribution and exist as parasites of plants, animals, and fungi and also as saprophytes (62). *Pythium* spp. cause damping off diseases such as root rots, seedling blights, and stem rots of many commercial crops and other plants (64). Under intense cultural practices in greenhouse production, *Pythium* spp. can cause significant crop losses. When root tips are attacked, plants become stunted and flower prematurely, negatively affecting the production cycle. The disease often kills plants, especially seedlings or cuttings. *Pythium* spp. are not true fungi in the phylogenetic sense but are mycelial organisms that belong to the family Pythiaceae of the order Peronosporales of the class Oomycetes and are related to the heterokont algae. Like true fungi, Oomycetes grow by extension of filamentous branching hyphae, obtain nutrients by absorption (osmotrophic), and reproduce via formation of asexual and sexual spores (26). However, Oomycetes possess

features such as cellulose in their cell walls, vegetative diploidy, heterokont flagellae (one tinsel and one whiplash), and tubular mitochondrial cristae that distinguish them from true fungi (64).

The life cycle of *Pythium* spp. is complex, including homothallic-heterothallic sexual and various asexual systems. Development of sexual spores called oospores involves the production of a female oogonium and a male antheridium that grows and attaches to the oogonium. Fertilization occurs when the antheridium produces a fertilization tube that penetrates into the oogonium and delivers the male nucleus to the egg. Following karyogamy, the resulting diploid zygote develops a thick, resistant wall, completing the process of oosporogensis (64). In addition to genetic recombination, the resistant oospores provide for long-term persistence of *Pythium* spp. in the soil environment, especially over long winter or dry seasons. These propagules are able to germinate only after undergoing a rest period and are frequently referred to as resting spores. Oospores germinate to produce single or multiple germ tubes that can directly infect host plants or form sporangia described below. A variety of factors influence the germination of oospores, including desiccation, temperature, pH, and presence of root or seed exudates (7, 30, 55, 59).

The asexual portion of the *Pythium* life cycle usually involves the production of sporangia. Sporangia may germinate either directly by producing a germ tube (direct germination) or may undergo cytoplasmic cleavage to form uninucleate, biflagellate zoospores (indirect germination). The zoospores are often held within a discharge vesicle that emerges from the sporangial pore and then bursts, freeing the zoospores. The role of a zoospore of a plant pathogen is to locate and encyst on the surface of a potential host. Energy for the swimming phase is derived from stored lipids (9); thus, a zoospore's life is ephemeral, ranging from just hours to a few days (32). The cyst typically germinates within an hour of locating a host,

producing either a germ tube that penetrates the host directly or an appressorium or appressorium-like structure. The mechanisms underlying root-targeting by phytopathogenic zoospores are complex and incompletely understood; the process may involve chemotaxis, chemically induced zoospore encystment, germ tube chemotropism, autoaggregation, auto-encystment, germ tube autotropism, electrotaxis to either anodic or cathodic regions of roots, electrically induced encystment, and zoospore pH taxis (26).

Many *Pythium* strains also produce asexual spore-like propagules called hyphal swellings (38, 45, 63). Hyphal swellings closely resemble sporangia and have often been identified as such (2, 10, 57). Germination of these propagules, however, is exclusively by production of germ tubes (no zoospores are produced). Though not generally referred to as resistant spores, hyphal swellings of at least some *Pythium* spp. are capable of surviving many months in soil, making them functionally analogous to chlamydospores (57). In fact, chlamydospores (thick-walled asexual spores derived form hyphal cells) have been described from at least two *Pythium* species (63), and it may be that the hyphal swellings of many more species are closer to chlamydospores (or at least more robust and more resistant) than generally recognized. Production of hyphal swellings does not appear to be correlated with production of other propagules; some *Pythium* species produce hyphal swellings in addition to sporangia and others produce only hyphal swellings; hyphal swelling may be the only spore-like propagules produced by *Pythium* strains that produce neither oospores nor sporangia (44, 63). Unlike the aerial, caducous conidia of many plant pathogenic fungi or sporangia of some other oomycete plant pathogens, the hyphal swellings of *Pythium* spp. are produced in infected host tissues or in the soil, generally remain attached to the parent hyphae, and are not adapted for aerial dispersal, although they can be disseminated in airborne soil particles or plant debris.

To rapidly detect and identify *Pythium* spp. responsible for plant diseases, specific isolation protocols are necessary. Early qualitative isolation methods involved various baiting techniques by placing living plant material in soil and vice versa. The infected plant material was then placed on agar media to isolate the pathogen. Selective media have also been used for the isolation of *Pythium* spp., incorporating polyene antibiotics combined with various chemicals, such as pentachloronitrobenzene, streptomycin, and rose bengal. Identification of individual *Pythium* spp. is then based on morphological and physiological differences (3, 30, 31, 48).

None of the above-described media are appropriate for all pathogenic *Pythium* spp., and none give an accurate estimation of total *Pythium* spp. in a sample (30). A variety of the plating methods fail to eliminate nonpathogenic *Pythium* spp. and some of the pathogenic species grow too slowly to be counted (56). Furthermore, there is a high potential for errors in morphological identification because of the great similarity in shapes and sizes of microscopic structures among species. Some taxonomic characteristics such as ornamentation of oogonia and heterothallism have been acquired or lost multiple times through evolution. The number of taxonomists who are capable to identifying Oomycetes to the species level is declining rapidly. A variety of molecular approaches have been developed to rapidly and accurately identify *Pythium* spp. from symptomatic or asymptomatic tissue. These molecular techniques include protein analysis, isozyme analysis, immunological methods, DNA probe, analysis by polymerase chain reaction (PCR) amplified rDNA, random amplified polymorphic DNA-PCR (RAPD-PCR), and restriction fragment length polymorphism (RELP) analysis of the internal transcribed spacer region of nuclear and mitochondrial DNA (23, 56, 62).

Molecular studies have also been employed to identify sources of inocula and track the movements of *Pythium* pathogens between and within greenhouse facilities (49). In a study examining sources of *Pythium* inoculum in greenhouse soils with no previous history of cultivation, approximately 7% of fallow soils were found to contain *Pythium* before being introduced to greenhouses. Other possible sources of *Pythium* include potting mixtures and contaminated soil adhering to cultivation equipment, grower's shoes, and reused irrigation pipes. Eighty-eight percent of the *Pythium* isolates from the different sources were of *Pythium aphanidermatum* (Edson) Fitzp (4). *Pythium* spp. have also been isolated from symptomless plants in greenhouses, with this genus being the third most common of the fungi and oomycetes isolated from cucumber roots (46).

Pythium species are also capable of spreading via irrigation water. Twenty-six pathogenic species of *Pythium* have been identified from irrigation water, including *P. aphanidermatum*, *Pythium irregulare* Buisman, and *Pythium ultimum* Trow (33). In a study examining *Pythium* species in plant samples, potting soil tests, and tests of irrigation water, *P. irregulare* was found in 45% of the plants samples, four of the five water samples, and three of the five potting soils. *P. aphanidermatum* was found in 29% of all the plant samples (49). The aforementioned *Pythium* species appear to be particularly problematic in greenhouses as *P. aphanidermatum* and *P. irregulare* are regularly isolated from greenhouse crops, with *P. aphanidermatum* causing the most deaths in one study (20).

Association of Insects with Plant Pathogens in Greenhouses

The association of insects with plant pathogens has been documented for more than a century. As early as 1891, M.B. Waite demonstrated that honeybees (*Apis* spp.) play a major

role in the movement of *Erwinia amylovora* (Burrill) Winslow et al., a bacterial pathogen that causes fire blight of apple and pear trees. In the greenhouse setting, aphids are considered to be one of the most serious pests of crops (67). Approximately 55% of arthropod disease vector species are aphids, and these insects are responsible for spreading a great number and variety of plant viruses, including the *Potyviruses*, *Luteoviruses*, *Cucumoviruses*, and *Closteroviruses* (50). Thrips, another group of serious greenhouse pests, are major vectors of *Tospoviruses*. Greenhouse flower crop selection and disease and insect management priorities have been greatly influenced by the fact that aphids and thrips transmit viruses (14). However, these key pests are not the only important vectors of greenhouse plant pathogens. Other insects, including some previously considered merely nuisance pests, also have been found in association with plant pathogens and implicated in disease transmission. Examples of such insects are fungus gnats, *Bradysia* spp., and shore flies, *Scatella spp*. Understanding the interactions of these insects with pathogens that are primarily soilborne is of particular significance, as aerial transmission would change the epidemiology of diseases from typical monocyclic to polycyclic patterns. The development of more appropriate disease management strategies may be warranted, depending on the nature of vector-pathogen relationships (16).

Fungus gnats have been reported in association with diseased plants for over a century (12). Mercier (47) showed that the fungus gnat *Sciara thomae* Scopoli carried *Claviceps* spp. spores from ergotized grain, both externally and internally. Spores that passed through the digestive tracts of *S. thomae* larvae retained viability. Graham and McNeill (27) observed that soybean plants damaged by *B. coprophila* larvae became infected with pathogens including *Pythium* spp. and *Fusarium* spp. It has also been shown that certain types of fungal spores can

adhere to the legs and bodies of adult fungus gnats and that these insects carry the mycopathogens into mushroom production facilities (11).

Several studies within the last few decades have revealed the association of plant pathogens with fungus gnats and shore flies. Kalb and Millar (39) found that adult *B. impatiens* from a greenhouse or growth chamber that contained alfalfa plants infected with Verticillium wilt were externally contaminated with spores of the fungus *Verticillium albo-atrum* Reinke & Berthold. Alfalfa plants caged with these insects became infected, whereas plants caged with *Trichoderma*-infested flies or with uninfested flies remained healthy.

Adult fungus gnats, *Bradysia* spp., and shore flies, *S. stagnalis*, associated with diseased roots of seedlings in a conifer nursery were found to be surface-contaminated with several fungi, including the seedling pathogens *Botrytis cinerea* and species of *Fusarium* and *Phoma*. The fact that the fungus gnats and shore flies yielded more *B. cinerea* (which causes gray mold) in summer than in winter supports their possible role in gray mold epidemiology since infection occurs in the summer (40, 53). In forest nurseries of South Africa, the fungus *Fusarium circinatum* Nirenberg & O'Donnell is the causal agent of a serious disease of pine seedlings. The association between fungus gnats and fungal pathogens led to questions whether these insects may be vectors of *F. circinatum* in South African forestry nurseries. Although the fungus gnat *Bradysia difformis* Frey was found in all nurseries surveyed, *F. circinatum* was not isolated from any of the collected fungus gnats (35).

In North America greenhouses, however, fungus gnats and shore flies have been shown to directly or indirectly facilitate infection by *Fusarium* spp. on a variety of crops. Fungus gnat larval feeding predisposed alfalfa (*Medicago sativa* L. 'DuPoits') and red clover (*Trifolium pratense* L. 'Pennscott') plants to infection by *Fusarium oxysporum* f.sp. *medicaginis* Snyder &

Hansen and *Fusarium roseum* Link., respectively (43). In a study involving *Fusarium oxysporum* f.sp. *radicis-lycopersici* Jarvis & Shoemaker, fungus gnat adults transported this pathogen from Petri dish culture and infected host plants to the roots and hypocotyls of healthy tomato (*Lycopersicon esculentum* Mill cv. Dombito) and pinto bean (*Phaseolus vulgaris* L.) plants. However, the method of pathogen acquisition by adult fungus gnats was not revealed. The presence of fungus gnat larvae in the media in which young tomato plants were grown did not increase the incidence of plant infection by *F. oxysporum* (24). El-Hamalawi and Stanghellini (18) found that both adult shore flies (*Scatella* spp.) and adult fungus gnats (*Bradysia* spp.) were attracted to and acquired (externally and/or internally) macroconidia of *Fusarium avenaceum* (Fr.) Sacc. produced on naturally infected lisianthus (*Eustoma grandiflorum* (Raf.) Shinners) stems and transmitted the macroconidia to healthy plants, resulting in plant death, or to an abiotic substrate (Komada's medium, KM). These insects can apparently be highly efficient as vectors, as evidenced by both the number of KM plates colonized by the pathogen (up to 68.5% within 18 hours) and the number of plants infected (75% within four days).

Another study on Fusarium wilt disease revealed that as few as 100 conidia ml^{-1} were sufficient to cause significant disease of Hiemalis begonias (*Begonia* x *hiemalis* Fotsch) by *Fusarium foetens* Schroers and that adult fungus gnats (*Bradysia* spp.) were efficient vectors of the pathogen (19). It is not likely, however, that control of fungus gnats and shore flies would completely control Fusarium crown and root rot disease in greenhouses because microconidia of *Fusarium* spp. disperse aerially (54). Nevertheless, control of these pests could reduce or slow the spread of *Fusarium* spp. in greenhouses (18).

Another fungal pathogen associated with fungus gnats and shore flies is *Thielaviopsis basicola* (Berkeley & Broome) Ferraris. In the case of this pathogen, abundant sporulation (production of chlamydospores) occurs on the hypocotyls of infected plants. Sporulation of the pathogen on the above-ground portion of the hypocotyl, often just above the soil line, provides an accessible source of inoculum for contamination of visiting insects. Stanghellini *et al.* (58) reported finding chlamydospores of *T. basicola* in the frass of adult and larval shore flies collected near naturally infected corn-salad plants (*Valerianella locusta* (L.) Betcke) obtained from a commercial greenhouse production facility. Approximately 95% of the adult flies and 85% of the larvae were internally infested with the pathogen. Pathogen-free adult shore flies were shown to obtain the pathogen when caged with naturally infected plants. Internally infested adults excreted the viable pathogen onto healthy seedlings, resulting in infection (58). Although the majority of chlamydospores observed in the intestinal tract of larvae and in frass excreted by adults and larvae were fragmented into individual cells, Patrick *et al*. (52) found that fragmentation of chlamydospores into separate cells may be important for germination. Thus, chlamydospore passage through shore fly guts and deposition on the lower stems of susceptible plants results in a high probability of infection (58).

Harris (28) showed that the fungus gnat *B. coprophila* can transmit *T. basicola* to pansy seedlings (*Viola* spp. hybrids) by larval and adult surface contamination or by viable spores surviving passage through the alimentary tract in the case of larvae. The percentage of seedlings infected by *T. basicola* was significantly reduced when inocula were obtained from insects reared on mixed fungal cultures of *T. basicola* and a potential antagonist, *Fusarium proliferatum* (Matsushima) Nirenberg compared to inocula obtained from insects reared on monocultures of *T. basicola*. Seedlings inoculated with frass from larvae reared on mixed *T. basicola* and *F.*

proliferatum cultures became infected with *T. basicola*, suggesting that the microconidia of *F. proliferatum* were destroyed by passage through the larval digestive tract, whereas the chlamydospores of *T. basicola* survived.

El-Hamalawi (16) took a closer look at insect transmission of soilborne pathogens by examining acquisition, retention, and distribution of *Verticillium dahliae* Kleb., *Fusarium oxysporum* f.sp. *basilici* Tamietti & Matta and *T. basicola* by shore flies. Adult insects were attracted to sporulating cultures of the aforementioned fungi, as well as infected plant tissues. The insects were contaminated with fungal particles both internally and externally. The time of acquisition was rapid, with fungal propagules detected in 10-20 minutes. After two hours of acquisition, 100% of frass deposits were infested with the fungi. The area over which the adult shore flies distributed the pathogens increased with increase in exposure time. The same trends of dispersal have also been demonstrated with fungus gnats in relation to the pathogens *V. dahliae*, *Fusarium acuminatum* Ellis & Everhart, and *T. basicola* (17). The mean number of colonies that developed on potato dextrose agar dishes from fungus gnats externally contaminated with the aforementioned fungi was 44, 38, and 46%, respectively. Since adult shore flies and fungus gnats are mobile, they may play an important role in carrying pathogen propagules over areas distant from the source of inoculum.

Fungus gnats and shore flies have also been implicated in the spread of oomycete plant pathogens (22, 25, 37). From a study with hydroponically grown cucumbers infected with *P. aphanidermatum*, Goldberg and Stanghellini (25) reported that larval and adult shore flies ingested oospores by feeding on diseased cucumber roots and excreted oospores that were capable of germinating. *P. aphanidermatum* was transmitted to healthy cucumber plants by naturally infested larval and adult shore flies. A high percentage of larvae and a low percentage

of adults were internally infested with the pathogen. To explain this discrepancy, Goldberg and Stanghellini (25) suggested that the adult shore flies "did not have access to infected roots," and that the "oospores observed in the guts of the adults likely reflected residual oospore populations retained after pupation." An alternative explanation, however, is simply that the adults chose to feed less on (or farther from) diseased roots than on surrounding algal mats that were only sparsely contaminated with oospores. These results are in contrast to the aerial transmission of *T. basicola* to corn-salad plants by shore flies since adults acquired the pathogen by direct feeding. In this case, a high percentage of the adult flies (95%) were internally infested with the pathogen (21, 58).

With regard to sciarid fungus gnats, Gardiner *et al*. (22) reported that larvae of *B. impatiens* were able to complete development on active cultures of three *Pythium* spp., and that oospores and encysted zoospores of these microbes were readily ingested by the larvae, survived passage through the digestive tract, and germinate normally after excretion. Jarvis *et al*. (37) showed in the laboratory that larvae of fungus gnats could ingest and excrete viable oospores of *P. aphanidermatum* and transmit the pathogen to healthy cucumber plants grown in rockwool, resulting in infection. Hyder *et al*. (36) confirmed that *P. aphanidermatum* oospores remain viable after passage through fungus gnat larval guts. They also observed survival of hyphal swellings of *P. splendens*, *P. sylvaticum*, and *P. ultimum* and demonstrated that *Pythium* propagules excreted by fungus gnat larvae were able to infect pepper seedlings in vitro. In the study by Jarvis *et al*. (37), however, adult fungus gnats did not ingest *Pythium*, and external transmission of *P. aphanidermatum* on the surface of adults could not be shown.

Despite the evidence of low potential for long-distance dissemination of *Pythium* spp. by fungus gnats, the perception has still persisted that these insects are important vectors of these

pathogens (36). In the current study, laboratory experiments were conducted to address the following objectives: 1) What are larval and adult fungus gnat attraction and oviposition responses to plants infected/infested with *Pythium* spp. versus non-infected/infested plants? 2) What is the potential for adult fungus gnats to acquire infectious propagules of *Pythium* spp. from diseased plants and transmit them to healthy plants under laboratory conditions highly favorable for infection? 3) Can natural variation in the biology of *Pythium* strains affect the likelihood of transmission by fungus gnats? 4) Does wounding damage inflicted by fungus gnat larvae affect the susceptibility of geranium seedlings by *Pythium aphanidermatum*?

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CHAPTER 2: Attraction and oviposition responses of the fungus gnat *Bradysia impatiens* to microbes and microbe-inoculated seedlings in laboratory bioassays

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ABSTRACT

Braun, S.E., Sanderson, J.P., Daughtrey, M.L., and Wraight, S.P. Attraction and oviposition responses of the fungus gnat *Bradysia impatiens* to microbes and microbe-inoculated seedlings in laboratory bioassays

Laboratory tests were conducted to examine preferences of *Bradysia impatiens* Johannsen (Diptera: Sciaridae) larvae and adults for various microbes associated with greenhouse crops. Fungus gnat larvae and adults exhibited a preference for cultures of *Pythium* spp. over the medium used to grow the pathogens. Larvae also exhibited a preference for geranium seedlings infected with pathogenic *Pythium* spp. (*P. aphanidermatum*, *P. ultimum*, and *P. irregulare*) over non-inoculated plants. Adult fungus gnats exhibited a strong ovipositional preference for the aforementioned *Pythium* spp. as well as a variety of other microorganisms, including the pathogenic fungus *Thielaviopsis basicola*, the geranium-infecting bacterium *Xanthomonas campestris* pv. *pelargonii*, the non-pathogenic species *P. torulosum* and *P. graminicola*, the pathogen-suppressive fungus *Trichoderma harzianum*, and the insect pathogenic fungus *Beauveria bassiana*. Our study demonstrates that fungus gnats are attracted to and/or stimulated to oviposit by a wide array of living microorganisms both in pure culture

and in association with plant seedlings. These findings have important implications with respect to the role of fungus gnats in plant disease epidemics.

INTRODUCTION

Fungivorous insects are abundant and include a variety of Collembola and larval and adult Coleoptera and Diptera that vary in the specificity of their fungal feeding. Among the Diptera, the dark-winged fungus gnats (Sciaridae) are diverse and speciose, and fungi are thought to make up an essential component of their diets (17, 26). Sciarids generally live in moist shady habitats; the larvae of most species live in fungi, but some inhabit soils, and a few are agricultural pests in field crops, greenhouses, or mushroom cellars (17, 33, 34). Kennedy (27) described sciarid adults as generally aphagous, although they have been reported to feed on liquids, including nectar (28), molasses (20), and "organic ooze" (33). In greenhouses, fungus gnat larvae feed primarily on decaying roots and stems of many plant species (17, 20, 23, 26). However, larvae of some pest species also sometimes consume apparently healthy root, stem, and leaf tissues of vascular plants in greenhouses (36).

In general, fungus gnat survival is reduced when fungal abundance is low (17). Kennedy (26) demonstrated that *Bradysia impatiens* (Johannsen) larvae developed more rapidly and exhibited greater survivorship when reared on fungal diets of *Alternaria tenuis* Nees or brewer's yeast (*Saccharomyces cerevisiae* Meyen ex E.C. Hansen) than on non-fungal diets of lima bean agar, potato dextrose agar, or agar-agar. Anas and Reeleder (1) also found that *Bradysia coprophila* (Comstock) larvae developed into fertile adults when raised on plants inoculated with *Botrytis porri* Buckw., *Rhizoctonia solani* Kühn, or *Sclerotinia minor* Jagger, but failed to survive on noninfected plants. Olson et al. (30) revealed that a significantly greater number of

Bradysia sp. adults emerged from soilless media composed of coconut coir or sphagnum peat moss when a food source of 1 g of yeast was added than from pure or sterilized soilless media.

Very little is known with respect to the capacity of sciarid fungus gnats to utilize microorganisms other than true fungi. In recent years, observations have pointed to a possible correlation between *Bradysia* fungus gnat infestations and root rot diseases caused by many common Oomycetes, especially *Pythium* spp. (11, 22). *Bradysia* and *Pythium* spp. seem to be constantly associated in greenhouses (personal observations, J.P. Sanderson & M.L. Daughtrey), and such observations have fostered the general perception that these insects are significant *Pythium* vectors (11, 22). In fact, however, potential interactions among fungus gnats, *Pythium* spp., and their host plants have not been thoroughly investigated (17). It may be that the close association between fungus gnats and diseased plants is based primarily on a strong attraction to microbial activity in diseased plant tissues. Fungus gnats thus may become associated with plants infected by microbes that they do not play a significant role in vectoring. The overall goal of the current study was to improve our understanding of the role of fungus gnats in the epidemiology of plant diseases, particularly in greenhouses where these insects are ubiquitious pests. The specific objective was to assess larval and adult fungus gnat attraction and oviposition responses to plants infected/infested with *Pythium* spp. versus non-infected/infested plants. Oviposition responses to other agriculturally important microbes and to live versus killed mycelial preparations of a common *Pythium* pathogen were also investigated.

MATERIALS AND METHODS

Insect Rearing. A laboratory colony of *B*. *impatiens* was established with adults collected from a greenhouse on the Cornell University campus. Adults and larvae were routinely reared in plastic containers (9.8 cm diam. x 6.2 cm depth; Pioneer Plastics, Dixon, KY) with lids bearing holes (5.5 cm diam.) covered with nylon mesh (95 µm) for ventilation. Each container was provided with 40 g of growing medium (Premier Pro-Mix® BX, Quakertown, PA) mixed with 10 g (dry weight) of ground pinto beans (Goya® Foods, Inc., Secaucus, NJ). The mix was then saturated with tap water, and 50 fungus gnat adults (predominately females) were introduced for egg production. Colony containers were maintained at 27 ± 1ºC and 14h:10h Light:Dark (L:D). In the following description of methods, these specific temperature/light conditions will be referred to as the "standard incubation conditions" or simply "standard conditions." New containers were established daily to provide a constant supply of all life stages. To obtain an even-aged cohort of third-instar fungus gnat larvae for use in bioassays, approximately 200 adult fungus gnats were collected from colony containers and released into a 45 cm x 45 cm x 45.5 cm cage in the laboratory at ambient temperature and allowed to oviposit on 90-mm-diam. Petri dishes (Becton Dickinson Labware, Franklin Lakes, NJ) that contained saturated cotton covered with a piece of black filter paper spread with a thin layer (approximately 1.6 g) of ground pinto beans (*Phaseolus vulgaris* L.). After 24 h, Petri dishes were removed from cages and the fungus gnat cultures maintained until the larvae completed development to the third-instar. Additional water and pinto beans were added to the dishes as needed. Third-instar larvae were readily identified on the surface of the filter paper and transferred to bioassay containers using a fine brush.

Plant Propagation. Geranium seeds (*Pelargonium* x *hortorum* L.H. Bailey 'Orbit White', Goldsmith Seeds®) were surface sterilized in a 0.5% sodium hypochlorite solution and germinated individually on top of a filter paper disk (42.5-mm diam.) saturated with 400 µl sterile distilled water (SDH_2O) in 47-mm diam. friction-lid Petri dishes (Fisher Scientific, Pittsburgh, PA, Cat. No. 09-720-501). The seeds were incubated in darkness for 3 days at room temperature. Seedlings were maintained under standard conditions and watered daily with 200 µl SDH_2O until used in bioassays. The seedlings were oriented, for the most part, horizontally in the Petri dishes (lying on the filter paper).

Seeds used in plug cell bioassays were planted in tap water-saturated Redi-earth Plug and Seedling Mix (Sun Gro Horticulture Canada Ltd., Vancouver, BC) within individual plug cells (ca. 14 ml volume) that were cut from 128-well plastic plug trays (Dillen Products/Myers Industries Inc., Middlefield, OH) and incubated in darkness at 23ºC until germination (4 days). Seedlings were maintained under standard conditions until used in bioassays.

Pathogen Maintenance. *Pythium aphanidermatum* (Edson) Fitzp strain Pa58 and *Pythium ultimum* Trow strain P4 were obtained from a culture collection maintained at Cornell University, Ithaca, NY (EBN lab), and *Pythium irregulare* Buis. strain Par 1 was obtained from a collection maintained at the Long Island Horticultural Research and Extension Center (LIHREC), Riverhead, NY (MLD lab). Cultures were grown in 60-mm-diam. Petri dishes on V8 juice agar (composed of 100 ml V8 juice, 400 ml H_20, 1.5 g $CaCO_3$, and 10 g Bacto™ Agar) and maintained in the dark at 27 ± 1ºC. For some experiments with *P. aphanidermatum*, cultures were grown in 60-mm-diam. Petri dishes on potato dextrose (PD) broth, comprising 6 g PD powder (MP Biomedicals, Solon, OH) in 250 ml SDH_2O, and maintained in the dark at 27 ± 1ºC.

One-week-old *Pythium* cultures were used in all experiments and for inoculating plants for periodic reisolation of the pathogens. Every two weeks the pathogens were reisolated from 11-day-old geranium seedlings that had been inoculated 3 days earlier by placing a 6-mm-diam. *Pythium*-colonized agar disk on the root tip. Each infected seedling was placed in a 90-mm-diam. Petri dish and covered with 2% molten water agar supplemented with the antibiotics rifampicin, penicillin G, and kanamycin (1.0, 1.0, and 2.5 ml/l SDH_2O, respectively and each 0.01 g/ml) (WARP). *Pythium* hyphae that grew rapidly through the agar were excised from the surface of the medium and transferred to a fresh WARP plate. *Pythium torulosum* Coker & P. Patt and *Pythium graminicola* Subramaniam (LIHREC strains Pt-1 and L-59, respectively, both of which are not pathogenic on geranium), were grown on V8 juice agar as described above.

Thielaviopsis basicola (Berk. & Br.) (obtained from LIHREC) was grown in the dark on PD agar (2% agar) at 24 ± 1ºC. One-week-old cultures of *T. basicola* were used in all experiments. Every two weeks the pathogen was reisolated from 18-day-old geranium seedlings that had been inoculated 10 days earlier as described above for *Pythium*-infected plants. *Xanthomonas campestris* (Brown) Dye strain 98-024 (obtained from D.W. Kalb of Cornell University, Ithaca, NY) was grown in the dark at 24 ± 1ºC. Two-day-old cultures of *X. campestris* were used in all experiments. *Trichoderma harzianum* strain T-22 and *Beauveria bassiana* (Balsamo) Vuillemin strain GHA cultures were grown in the dark on either potato dextrose broth or potato dextrose agar at 24 ± 1ºC.

Infection of Seedlings. Eight-day-old seedlings in the previously described friction-lid Petri dishes were inoculated by placing a 6-mm-diam. agar disk colonized by either *P. aphanidermatum*, *P. ultimum*, or *P. irregulare* directly onto the root tips. The seedlings were

then maintained under standard conditions and watered daily (200 µl SDH_2O) until they were 11 days old and exhibited signs of infection, including wilting and water-soaking that made the tissue appear darker green in color. Using the same protocol, additional seedlings were inoculated with *T. basicola* or *X. campestris* and incubated at 24 ± 1ºC under the standard light regime. Seedlings infected with *T. basicola* were maintained until they were 18 days old and exhibited black and water-soaked roots; *X. campestris*-infected seedlings were maintained until they were 12 days old and exhibited wilting and yellowing. Seedlings were also inoculated with the non-plant-pathogenic microbes *P. graminicola*, *P. torulosum*, *T. harzianum*, or *B. bassiana* as described above and maintained under standard conditions until they were 11 days old.

Seven-day-old seedlings in plug cells were inoculated by placing a 6-mm-diam. agar disk of either *P. aphanidermatum* or *T. harzianum* in contact with the crown of the plant. Seedlings were watered (with 2 ml tap water) after inoculation and maintained under standard conditions until the plants were 10 days old. Inoculation disks were removed from seedlings prior to the initiation of experiments.

Larval Choice: *Pythium*-colonized agar disks.

Assays were conducted to determine fungus gnat larval preference for *Pythium* spp. All assays were conducted on the same lab bench under room temperature conditions (25 ± 1ºC). Each assay was conducted on the wet surface of a 90-mm-diam. disk of filter paper (saturated with 1.5 ml SDH_2O) lining a Petri-dish lid with inside diameter of 91 mm (Becton Dickinson Labware, Franklin Lakes, NJ). Four 12-mm-diam. V8 agar disks, one colonized with *P. aphanidermatum*, one with *P. ultimum*, one with *P. irregulare,* and one un-colonized disk were positioned around the perimeter of the filter paper, equidistant from one another and from the center of the dish

(centers of the disks were ca. 5.2 cm apart and 3.7 cm from the center of the dish). In each dish, the locations to which the four disks were randomly assigned corresponded to the extreme back, right, front, and left from the fixed viewpoint of the observer (0/360º, 90º, 180º, and 270º). Approximately 20 min. after placement of the disks, one third-instar fungus gnat larva was deposited (oriented indiscriminately) in the center of the arena using a fine paintbrush, and the arena was covered with the dish bottom (87-mm inside diam.). Movements of the larva were then visually monitored. A choice was scored upon first contact between the larva and one of the disks, and the assay was terminated after 30 min. Larvae that did not contact a disk within 30 min. were classified as non-responders. A total of 71 replicate assays were conducted on three different days (23–24 assays/day) using different batches of fungus gnats.

Larval Choice: *Pythium*-infected vs. non-infected geranium seedlings.

Fungus gnat larval preference for *Pythium*-infected vs. non-infected geranium seedling roots was assessed using the same arena and general protocol as described above for the agar disk assays. Pieces of infected root tip (2 cm in length) were cut from 11-day-old geranium seedlings infected with either *P. aphanidermatum*, *P. ultimum*, or *P. irregulare* as previously described. One root tip from each of the three *Pythium*-infected seedlings and one from an 11-day-old non-infected seedling were randomly assigned to the four previously described positions on the wet filter paper (root pieces were oriented approximately parallel to the wall of the dish). One third-instar fungus gnat larva was placed in the center of the arena, and its movements were visually monitored. A choice was scored upon first contact between the larva and one of the root tips, and the assay was terminated after 90 min. Larvae that did not contact a root tip within 90 min. were

considered non-responders. Eighty-two replicate assays were conducted on four different days (ca. 10–24 assays/day).

Adult Choice: *Pythium*-colonized agar disks.

Assays were conducted to determine fungus gnat adult preference for disks of *P. aphanidermatum*. Assays were conducted in 150 x 25-mm Petri dishes (Becton Dickinson Labware, Franklin Lakes, NJ). One disk of *P. aphanidermatum* and one of V8 agar alone (all disks 12-mm-diam.) were placed opposite one another equidistant from the center of the Petri dish. The centers of the disks were ca. 6 cm from the center of the dish and 12 cm apart. In each assay, the fungus-colonized disk was first randomly assigned to one of the four positions described for the larval-choice assays, and then the uncolonized agar disk was placed in the opposite location. The agar disks were placed directly on the plastic dish (arena without filter paper). Two-day-old adult female fungus gnats were collected individually into small (20 ml) glass vials and chilled at 4ºC for 10 min. The vial was then inverted over the center of the arena and tapped sharply to dislodge the test subject. The protocol then followed that of the larval choice assays, except that the dishes were monitored for a maximum of 2 h. Seventy-six replicate assays were conducted on four different days (17–20 assays/day).

Adult Choice: Oviposition on *Pythium*-infected, heat-killed, or non-infected plants.

Thirty replicate assays were conducted to determine fungus gnat ovipositional preference for *Pythium*-infected, non-infected, or heat-killed geranium seedlings using the 90-mm-diam. Petri dishes with wet filter paper as described for the larval choice tests. Treatments included 11-day-old seedlings that were either non-infected, freshly killed via a 30 sec. microwave treatment

(microwave oven set on high; Model R-120DK, Sharp Electronics, Mahwah, NJ), or infected with *Pythium* (*P. aphanidermatum*, *P. ultimum*, or *P. irregulare*). One *Pythium*-infected, one heat-killed, and one non-infected seedling were placed randomly in parallel non-overlapping positions in the Petri dish, and one mated pair of 2-day-old fungus gnats (one male and one female) was added in the middle at the base of the dish. The dishes were sealed with Parafilm,® placed randomly on trays, and maintained under standard incubation conditions for 36 h. Eggs oviposited on the seedlings and filter paper were then enumerated. Each *Pythium* treatment (individual *Pythium* species) was replicated 10 times. Ten replicate control assays also compared preference for *P. aphanidermatum*-infected seedlings vs. seedlings sham-inoculated with V8 agar.

Oviposition assays in all additional experiments to be described below were based on the above-described protocol, unless otherwise indicated. In those assays testing only two substrates in addition to the filter paper surface, the substrates were randomly assigned to opposite positions in the arena as described for the experiments investigating adult fungus gnat choice of *Pythium*-colonized agar disks. Assays in all oviposition experiments were conducted on two or three different days, with an average of ca. 5 assays conducted each day.

Adult Choice: Oviposition on dead *Pythium* vs. live *Pythium* or healthy geranium seedlings. A series of assays was conducted to determine fungus gnat ovipositional preference for live *vs.* dead (dried or frozen) *P. aphanidermatum* and live or dead *P. aphanidermatum* vs. non-infected 7-day-old geranium seedlings. Mats of *P. aphanidermatum* mycelium were removed from the surface of PD broth, washed 3 times with SDH_2O, and cut into 1 x 1 cm pieces. Subsets of these pieces were then treated (killed) either by drying for 2 h in an open Petri dish in a sterile laminar

flow hood or by freezing for 3 h at -18ºC; non-viability of the dried/frozen pieces was confirmed by incubation on V8 agar. Two of the above-described substrates were randomly assigned to opposite positions in each assay arena. The SDH_2O used for filter paper saturation contained the antibiotic gentamicin sulfate at a concentration equivalent to 0.1 mg gentamicin base/ml to prevent bacterial growth over the course of the assays. Substrate combinations and numbers of assays (in parentheses) included: live vs. dried *Pythium* (18), live vs. frozen *Pythium* (13), live *Pythium* vs. non-infected plant (15), dried *Pythium* vs. non-infected plant (12), and frozen *Pythium* vs. non-infected plant (13). An additional 10 assays testing live vs. frozen *Pythium* were conducted without addition of gentamicin.

Adult Choice: Oviposition under no-choice conditions.

A series of assays was conducted to compare rates of oviposition on treated seedlings in no-choice assays. In these assays, a single non-infected, microwave-killed, or *Pythium*-infected 11-day-old geranium seedling was placed in the center of the Petri dish, and a pair of fungus gnats was added. Assays included non-infected seedlings (11), microwave-killed seedlings (11), and seedlings infected with *P. ultimum* (10), *P. irregulare* (10), or *P. aphanidermatum* (11).

Adult Choice: Oviposition on fungus- or bacterium-infected vs. non-infected plants.

Twenty-two replicate assays were conducted with *T. basicola*-infected geranium seedlings to determine fungus gnat ovipositional preference for fungus-infected vs. non-infected 18-day-old geranium seedlings, and 20 replicate assays were conducted to determine preference for *Xanthomonas campestris*-infected vs. non-infected 12-day-old geranium seedlings.

Adult Choice: Oviposition on non-pathogenic *Pythium* spp. vs. non-infected plants.

Assays were conducted with *P. torulosum*-inoculated geranium seedlings (11 assays) and *P. graminicola*-inoculated geranium seedlings (10 assays) to compare fungus gnat ovipositional preference for geranium seedlings inoculated with *Pythium* spp. non-pathogenic on geranium versus non-inoculated plants. Controls (11 assays) compared preference for seedlings sham-inoculated with V-8 agar vs. non-inoculated seedlings. Either one 11-day-old *P. torulosum*-inoculated, *P. graminicola*-inoculated, or V8 agar-inoculated geranium seedling and one 11-day-old non-inoculated geranium seedling were randomly assigned to opposite positions in the assay arena. Both *P. torulosum* and *P. graminicola* were re-isolated from stems and roots of inoculated seedlings at the end of the assays.

Adult Choice: Oviposition on beneficial microbes vs. non-infected plants.

Oviposition assays were conducted to assess the attractiveness of two commercially-available beneficial microbes, the mycoparasite *Trichoderma harzianum*, and the insect pathogen *Beauveria bassiana*. Fungus gnat ovipositional preference was determined for live *T. harzianum* or *B. bassiana vs*. non-infected 7-day-old geranium seedlings and *T. harzianum* or *B. bassiana*-inoculated *vs.* non-infected 11-day-old geranium seedlings. Mats of *T. harzianum* or *B. bassiana* were removed from the surface of PD broth, washed 3 times with SDH_2O, and cut into 1 x 1 cm pieces. Two of the above-described substrates were randomly assigned to opposite positions in each assay arena. Substrate combinations and numbers of assays (in parentheses) included: live *T. harzianum* vs. non-infected plant (19), *T. harzianum*-inoculated plant vs. non-inoculated plant (12), live *B. bassiana* vs. non-infected plant (16), and *B. bassiana*-inoculated plant vs. non-inoculated plant (12). An additional 16 assays testing PD agar-inoculated plants vs. non-

inoculated plants were conducted to determine if fungus gnats exhibit a preference for the inoculation medium. Both *T. harzianum* and *B. bassiana* were re-isolated from stems and roots of inoculated seedlings at the end of the assays.

Adult Choice: Oviposition on plants grown in plug cells

Assays were conducted with *P. aphanidermatum*-infected geranium seedlings (14 assays) and *T. harzianum*-inoculated geranium seedlings (10 assays) to compare fungus gnat ovipositional preference for plug cell-grown seedlings inoculated with microorganisms compared to plug cell-grown sham-inoculated seedlings. Assays were conducted in plastic containers (9.8 cm diameter x 6.2 cm depth; Pioneer Plastics, Dixon, KY) with lids. Either one 10-day-old *P. aphanidermatum*-inoculated and one V8 agar-inoculated geranium seedling or one 10-day-old *T. harzianum*-inoculated and one potato dextrose agar-inoculated geranium seedling were randomly assigned to opposite positions in each assay arena. One mated pair of 2-day-old fungus gnats (one male and one female) was added. Each plug cell was watered once with 1 ml tap water, and the containers were maintained under standard incubation conditions for 36 h. Both *P. aphanidermatum* and *T. harzianum* were re-isolated from inoculated seedlings and from the plug mix at the end of the experiment.

Statistical Analyses.

All statistical tests were conducted using the JMP® software version 7.0 (31). It was generally not possible to conduct large numbers of assays at one time (especially with the multiple species of pathogens and other microbes), and rigorous comparisons among the various assays is therefore not possible. Our analyses are thus focused on the responses to the different substrates tested

within assays. Proportions of larval or adult fungus gnats responding (attracted) to the various substrates within assays were initially tested for differences across test days by heterogeneity chi-square (X^2) analysis, and differences among proportions of the test population attracted to the various substrates were then examined via conventional goodness-of-fit X^2 analysis testing frequencies observed versus frequencies expected from a show of no preference (equal numbers of gnats attracted to each substrate) (32, 37). Alpha in all tests was set at 0.05.

Egg counts from the adult oviposition experiments were analyzed as repeated measures, with each female confined (confronted simultaneously) with multiple substrates. We opted to use the MANOVA approach. All percentages were arcsine transformed.

In experiments with more than two treatments, means comparisons were made via multiple pairwise tests (X^2 goodness-of-fit or repeated measures MANOVA) with sequential Bonferroni adjustments to maintain the experimentwise alpha level at 0.05 (32). No more than four means were directly compared in any test.

RESULTS

Larval Choice: *Pythium*-colonized agar disks.

The heterogeneity chi-square test indicated no differences in responses across days ($X^2_{[2]} = 3.20$, $P > 0.05$), and data from all replicates were therefore pooled. No differences in attractiveness were observed among the substrates colonized with the three *Pythium* spp. ($X^2_{[2]} = 4.53$, $P = 0.10$). The *Pythium*-colonized substrates (all *Pythium* species combined) attracted nearly 97% of all responding fungus gnats, with the V8 agar alone attracting < 4% (Table 2.1). Seventeen percent of the larvae (12/71) did not choose a disk within 30 min (Table 2.1).

Larval Choice: *Pythium*-infected vs. non-infected geranium seedlings.

As observed in the tests with agar disks, response was homogeneous across days, and data from all replicates were pooled ($X^2_{[3]} = 3.53$, $P > 0.05$). Overall results were also very similar to those obtained with agar disks: geranium roots infected with the three *Pythium* spp. were equally attractive ($X^2_{[2]} = 1.41$, $P = 0.49$); 94% of the responding larvae were attracted to the *Pythium*-infected roots vs. only 6% to the non-inoculated roots (Table 2.2). Twenty-one percent of larvae (17/82) did not choose a seedling within 90 min.

Adult Choice: *Pythium*-colonized agar disks.

Results did not vary significantly across days ($X^2_{[3]} = 0.24$, $P > 0.05$), and data from all replicates were pooled. Among 58 responding adult fungus gnats, a significantly greater number (64%) were attracted to the *P. aphanidermatum*-colonized disks vs. the disks of V8 agar alone ($X^2_{[1]} = 4.41$, $P = 0.036$). Among all flies tested, 24% (18/76) did not choose either disk within 2 h (Table 2.1).

Adult Choice: Oviposition on *Pythium*-infected, heat-killed, or non-infected plants.

There was a highly significant effect of oviposition substrate on the number of eggs laid in each assay (P values < 0.0001) (Table 2.3), and the overall oviposition responses were similar in the assays with the three different *Pythium* species. Approximately 86, 89, and 92% of all eggs were deposited on the *P. aphanidermatum*-, *P. irregulare*-, and *P. ultimum*-infected seedlings, respectively, whereas low, equivalent numbers of eggs were laid on the non-infected live and killed seedlings. In the control assay, < 3% of all eggs were deposited on the sham-inoculated seedlings.

Table 2.1. Number of fungus gnats attracted to *Pythium*-colonized vs. non-colonized V8 agar disks.

Assay – substrates tested	Number of fungus gnats [a]	Percent of total responses [b]
Larval fungus gnats		
Pythium aphanidermatum-colonized agar	25	42.4 a
Pythium irregulare-colonized agar	20	33.9 a
Pythium ultimum-colonized agar	12	20.3 a
Non-colonized agar	2	3.4 b
Total responding	59	100
Adult fungus gnats		
Pythium aphanidermatum-colonized agar	37	63.8 a
Non-colonized agar	21	36.2 b
Total responding	58	100

[a] Number of fungus gnats that selected each substrate (excluding 12 larvae that were non-responsive (did not make contact with a test substrate) within 30 min. and 18 adults that were non-responsive within 2 h).

[b] Larval assays: percentages followed by same letter are not significantly different (sequential Bonferroni test, experimentwise alpha = 0.05); adult assays: percentages followed by a different letter are significantly different (chi-square goodness-of-fit test, alpha = 0.05).

Table 2.2. Number of fungus gnat larvae attracted to *Pythium*-infected vs. non-infected geranium roots.

Treatment	Number of larvae [a]	Percent of total responses [b]
Pythium aphanidermatum-infected root	23	35.4 a
Pythium irregulare-infected root	16	24.6 a
Pythium ultimum-infected root	22	33.8 a
Non-infected root	4	6.2 b
Total responding	65	100

[a] Excluding 17 larvae that were non-responsive (did not make contact with a geranium root) within 90 min.

[b] Percentages followed by same letter are not significantly different (sequential Bonferroni test, experimentwise alpha = 0.05).

Table 2.3. Oviposition by fungus gnats on *Pythium*-infected vs. non-infected (healthy) geranium seedlings.

Assay – substrates tested	Number of replicate assays	Mean number of eggs	Percentage of eggs [b]		MANOVA F-test
Pythium aphanidermatum-infected seedling	10	46.3 ± 10.1	85.9 ± 6.5	a	$F_{[3,7]} = 51.1$
Non-infected seedling		0.9 ± 0.41	2.1 ± 0.8	b	$P < 0.0001$
Non-infected, microwave-killed seedling		1.9 ± 1.32	3.7 ± 2.6	b	
Filter paper		2.7 ± 1.87	8.3 ± 6.2	b	
Pythium irregulare-infected seedling	10	62.2 ± 6.37	88.7 ± 4.7	a	$F_{[3,7]} = 42.0$
Non-infected seedling		5.4 ± 3.14	6.1 ± 3.5	b	$P < 0.0001$
Non-infected, microwave-killed seedling		1.6 ± 0.79	3.4 ± 2.1	b	
Filter paper		1.3 ± 0.67	1.8 ± 0.8	b	
Pythium ultimum-infected seedling	10	58.2 ± 9.8	91.8 ± 3.5	a	$F_{[3,7]} = 43.2$
Non-infected seedling		1.2 ± 0.68	2.7 ± 1.9	b	$P < 0.0001$
Non-infected, microwave-killed seedling		0.9 ± 0.48	2.5 ± 1.6	b	
Filter paper		1.8 ± 1.29	2.9 ± 2.3	b	
Pythium aphanidermatum-infected seedling	10	60.4 ± 10.1	88.5 ± 5.9	a	$F_{[3,8]} = 76.2$
Sham-inoculated seedling [a]		1.6 ± 0.95	2.5 ± 1.4	b	$P < 0.0001$
Filter paper		4.7 ± 2.39	9.0 ± 5.0	b	

[a] Healthy geranium seedling sham-inoculated with V-8 agar.

[b] Mean percentage of eggs (± SE) laid on each substrate (one female fungus gnat per replicate assay). Means within each assay followed by the same letter are not significantly different (sequential Bonferroni test; experimentwise alpha = 0.05).

Adult Choice: Oviposition on dead *Pythium* vs. live *Pythium* or healthy geranium seedlings.

In each assay there was a highly significant effect of oviposition substrate on the number of eggs laid (all P values ≤ 0.0003) (Table 2.4). Across all assays with desiccation- or freeze-killed *Pythium* mycelium (with and without gentamicin) vs. live *Pythium* mycelium, only 2–5% of all eggs were deposited on the dead mycelium. In assays of killed *Pythium* vs. healthy geranium seedlings, significantly more eggs (75–79%) were deposited on the seedlings than on the killed *Pythium* (3–7%) or filter paper (18%). In assays of live *Pythium* mycelium vs. healthy geranium seedlings, significantly more eggs (62%) were deposited on the live *Pythium* than on the seedlings (26%) or filter paper (12%).

Adult Choice: Oviposition under no-choice conditions.

Results of assays in which fungus gnats were presented with just a single substrate (either a *Pythium*-infected, a non-infected, or a microwave-killed seedling) are presented in Table 2.5. Seedlings infected with *P. aphanidermatum*, *P. irregulare*, and *P. ultimum* received 86, 86, and 81% of all eggs, respectively, with the remaining eggs in each assay being deposited on the wet filter paper. When confronted with the choice between non-infected (healthy) seedlings and filter paper, the fungus gnats deposited statistically equivalent numbers on each substrate (44 vs. 57%). In contrast, microwave-killed seedlings attracted < 7% of egg deposition.

Adult Choice: Oviposition on fungus- or bacterium-infected vs. non-infected plants.

There was a highly significant effect of oviposition substrate on the number of eggs laid in each assay (*P* values ≤ 0.0002) (Table 2.6), and the overall oviposition responses were similar in the assays with fungal and bacterial plant pathogens. Approximately 80 and 62% of all eggs were

Table 2.4. Effects of *Pythium aphanidermatum* viability (live vs. dead) on attractiveness to ovipositing fungus gnats.

Assay – substrates tested [a]	Number of replicate assays	Mean number of eggs	Percentage of eggs [b]	MANOVA F-test
Live *Pythium*	18	50.8 ± 8.68	82.3 ± 5.0 a	$F_{[2,16]} = 49.7$
Desiccation-killed *Pythium*		1.1 ± 0.51	4.6 ± 2.8 b	$P < 0.0001$
Filter paper		6.4 ± 2.16	13.1 ± 3.3 c	
Live *Pythium*	13	55.2 ± 7.65	85.4 ± 4.9 a	$F_{[2,11]} = 79.5$
Freeze-killed *Pythium*		1.1 ± 0.47	2.6 ± 1.2 b	$P < 0.0001$
Filter paper		5.4 ± 1.94	12.0 ± 4.7 b	
Live *Pythium*	10	30.6 ± 4.39	69.7 ± 5.4 a	$F_{[2,8]} = 34.5$
Freeze-killed *Pythium*		2.8 ± 1.31	5.1 ± 2.2 b	$P = 0.0001$
Filter paper (without antibiotic)		10.8 ± 1.9	25.2 ± 4.7 c	
Live *Pythium*	15	23.7 ± 3.26	61.6 ± 7.3 a	$F_{[2,13]} = 16.3$
Geranium seedling		12.6 ± 3.94	26.3 ± 6.8 b	$P = 0.0003$
Filter paper		5.2 ± 1.28	12.0 ± 2.5 b	
Desiccation-killed *Pythium*	12	1.8 ± 0.95	6.9 ± 4.8 a	$F_{[2,10]} = 21.0$
Geranium seedling		31.0 ± 5.11	75.4 ± 6.0 b	$P = 0.0003$
Filter paper		6.8 ± 1.77	17.7 ± 3.9 c	
Freeze-killed *Pythium*	13	0.9 ± 0.42	3.1 ± 1.5 a	$F_{[2,11]} = 82.5$
Geranium seedling		37.2 ± 8.18	78.6 ± 4.7 b	$P < 0.0001$
Filter paper		6.2 ± 1.67	18.3 ± 4.4 c	

[a] Substrates included 1 x 1 cm mats of live or dead *Pythium aphanidermatum* mycelium, healthy (non-infected) 7-day-old geranium seedlings, and water-saturated filter paper; the filter paper substrate (upon which all other substrates were placed) was saturated with water containing the antibiotic gentamycin in all but the indicated series of assays.

[b] Mean percentage of eggs (± standard error) deposited on each substrate in each assay (one female fungus gnat per replicate assay). Means within each assay followed by the same letter are not significantly different (sequential Bonferroni test; experimentwise alpha = 0.05).

Table 2.5. Oviposition by fungus gnats on filter paper vs. *Pythium*-infected geranium seedlings, non-infected seedlings, or microwave-killed seedlings.

Assay – substrates tested	Number of replicate assays	Number of eggs [a]	Percentage of eggs [a]	MANOVA F-test
Pythium aphanidermatum-infected seedling	11	44.9 ± 6.68	85.5 ± 5.7 a	$F_{[1,10]} = 20.1$
Filter Paper		7.1 ± 3.41	14.5 ± 5.7 b	P = 0.0003
Pythium irregulare-infected seedling	10	50.5 ± 7.95	86.4 ± 4.7 a	$F_{[1,9]} = 39.6$
Filter Paper		5.4 ± 1.18	13.6 ± 4.7 b	P = 0.0001
Pythium ultimum-infected seedling	10	58.5 ± 8.03	80.7 ± 6.6 a	$F_{[1,9]} = 17.0$
Filter Paper		11.9 ± 4.16	19.3 ± 6.6 b	P = 0.003
Non-infected (healthy) seedling	11	25.7 ± 8.19	43.5 ± 12.6 a	$F_{[1,10]} = 0.32$
Filter Paper		35.7 ± 9.6	56.5 ± 12.6 a	P = 0.58
Microwave-killed seedling	11	2.2 ± 0.96	6.8 ± 3.1 a	$F_{[1,10]} = 84.5$
Filter Paper		57.1 ± 9.18	93.2 ± 3.1 b	P < 0.0001

[a] Mean percentage eggs (± standard error) deposited on each substrate in each assay (one female fungus gnat per replicate assay). Means within each assay followed by the same letter are not significantly different (MANOVA F-test; alpha = 0.05).

Table 2.6. Oviposition by fungus gnats on *Thielaviopsis basicola*- or *Xanthomonas campestris*-infected vs. non-infected (healthy) geranium seedlings.

Assay – substrates tested	Number of replicate assays	Mean number of eggs	Percentage of eggs [a]		MANOVA F-test
Thielaviopsis basicola-infected seedling	22	40.1 ± 4.4	80.1 ± 4.4	a	$F_{[2,20]} = 38.1$
Non-infected seedling		4.8 ± 1.2	11.0 ± 3.0	b	$P < 0.0001$
Filter paper		3.9 ± 1.2	8.8 ± 2.8	b	
Xanthomonas campestris-infected seedling	20	35.0 ± 5.9	62.2 ± 7.7	a	$F_{[2,18]} = 14.2$
Non-infected seedling		13.0 ± 4.0	25.7 ± 7.2	b	$P = 0.0002$
Filter paper		6.3 ± 1.6	12.2 ± 2.9	b	

[a] Mean percentage of eggs (± standard error) deposited on each substrate in each assay (one female fungus gnat per replicate assay). Means within each assay followed by the same letter are not significantly different (sequential Bonferroni test; experimentwise alpha = 0.05).

deposited on the *T. basicola*-, and *X. campestris*-infected seedlings, respectively, whereas lower numbers of eggs were laid on the non-infected seedlings (11–26%) and filter paper (9–12%).

Adult Choice: Oviposition on non-pathogenic *Pythium* spp. or non-infected plants.

There was a highly significant effect of oviposition substrate on the number of eggs laid in each assay (*P* values $\leq$ 0.007) (Table 2.7), and the overall oviposition responses were similar in the assays with the two different *Pythium* species. Approximately 63 and 86% of all eggs were deposited on the *P. torulosum*- and *P. graminicola*-inoculated seedlings, respectively, whereas lower numbers of eggs were laid on the non-inoculated seedlings (10–21%) and filter paper (4–16%). When confronted with the choice between sham-inoculated vs. non-inoculated seedlings, the fungus gnats deposited statistically equivalent numbers on each substrate (46 vs. 51%).

Adult Choice: Oviposition on beneficial microbes or non-infected plants.

In each assay there was a highly significant effect of oviposition substrate on the number of eggs laid (all P values $\leq$ 0.004) (Table 2.8). Across all assays with live *Trichoderma* or *Beauveria* mycelia, significantly more eggs were laid on the mycelia (60 and 77%, respectively) than on the non-inoculated seedlings (29 and 18%) or filter paper (11 and 5%). In assays with *Trichoderma*- or *Beauveria*-inoculated vs. non-inoculated seedlings, significantly more eggs were laid on fungus-inoculated seedlings (85 and 80%, respectively) than on non-inoculated seedlings (8 and 14%) or filter paper (7 and 6%). When confronted with the choice between sham-inoculated and non-inoculated seedlings, the fungus gnats deposited statistically equivalent numbers on each substrate (35 vs. 54%).

Table 2.7. Oviposition by fungus gnats on *Pythium torulosum*-, *P. graminicola*-, or V8 agar-inoculated vs. non-inoculated geranium seedlings.

Assay – substrates tested	Number of replicate assays	Mean number of eggs	Percentage of eggs [a]	MANOVA F-test
Pythium torulosum-inoculated seedling	11	32.5 ± 5.0	62.7 ± 7.4 a	$F_{[2,9]} = 9.1$
Non-inoculated seedling		10.8 ± 3.7	21.4 ± 7.6 b	P = 0.007
Filter paper		8.7 ± 2.9	16.0 ± 4.0 b	
Pythium graminicola-inoculated seedling	10	75.0 ± 9.2	85.8 ± 5.6 a	$F_{[2,8]} = 54.5$
Non-inoculated seedling		9.7 ± 4.8	10.0 ± 4.7 b	P < 0.0001
Filter paper		3.5 ± 1.2	4.2 ± 1.4 b	
V8 agar-inoculated seedling	11	39.3 ± 12.1	46.3 ± 13.8 a	$F_{[2,9]} = 48.5$
Non-inoculated seedling		28.2 ± 7.5	51.3 ± 13.4 a	P < 0.0001
Filter paper		1.9 ± 1.2	2.4 ± 1.5 b	

[a] Mean percentage of eggs (± standard error) deposited on each substrate in each assay (one female fungus gnat per replicate assay). Means within each assay followed by the same letter are not significantly different (sequential Bonferroni test; experimentwise alpha = 0.05).

Table 2.8. Oviposition by fungus gnats on non-inoculated geranium seedlings vs. *Trichoderma harzianum*- or *Beauveria bassiana*-inoculated seedlings or culture mats.

Assay – substrates tested [a]	Number of replicate assays	Mean number of eggs	Percentage of eggs [b]		MANOVA F-test
Live *Trichoderma*	19	36.2 ± 5.3	60.0 ± 6.9	a	$F_{[2,17]} = 18.0$
Non-inoculated seedling		19.1 ± 4.0	28.8 ± 4.9	b	$P < 0.0001$
Filter paper		6.5 ± 1.8	11.2 ± 3.2	c	
Trichoderma-inoculated seedling	12	42.5 ± 4.7	85.1 ± 4.2	a	$F_{[2,10]} = 36.5$
Non-inoculated seedling		4.7 ± 2.1	7.9 ± 3.7	b	$P < 0.0001$
Filter paper		3.8 ± 1.8	6.9 ± 2.7	b	
Live *Beauveria*	16	35.6 ± 4.2	77.4 ± 6.0	a	$F_{[2,14]} = 62.0$
Non-inoculated seedling		9.4 ± 3.1	17.8 ± 5.2	b	$P < 0.0001$
Filter paper		2.3 ± 0.7	4.7 ± 1.5	c	
Beauveria-inoculated seedling	12	36.4 ± 6.0	80.0 ± 6.2	a	$F_{[2,10]} = 34.4$
Non-inoculated seedling		6.2 ± 2.3	14.0 ± 5.6	b	$P < 0.0001$
Filter paper		2.0 ± 0.6	6.1 ± 1.9	b	
Potato dextrose agar (PDA)-inoculated seedling	16	14.6 ± 4.5	34.6 ± 9.0	ab	$F_{[2,14]} = 8.59$
Non-inoculated seedling		25.9 ± 6.5	53.5 ± 10.0	a	$P = 0.0037$
Filter paper		4.2 ± 1.5	11.9 ± 4.3	b	

[a] Substrates included 1 x 1 cm mats of live *Trichoderma harzianum* or *Beauveria bassiana* mycelium, *T. harzianum*-, *B. bassiana*-, or PDA-inoculated geranium seedlings, healthy (non-infected) geranium seedlings, and water-saturated filter paper.

[b] Mean percentage of eggs (± standard error) deposited on each substrate in each assay (one female fungus gnat per replicate assay). Means within each assay followed by the same letter are not significantly different (sequential Bonferroni test; experimentwise alpha = 0.05).

Adult Choice: Oviposition on plants grown in plug cells.

There was a significant effect of oviposition substrate on the number of eggs laid in each assay (*P* values $\leq$ 0.03) (Table 2.9), and the overall oviposition responses were similar in the assays with the *P. aphanidermatum* and *T. harzianum*. Approximately 72 and 79% of all eggs were deposited on the *P. aphanidermatum*-infected and *T. harzianum*-inoculated seedlings, respectively, whereas lower numbers of eggs were laid on the sham-inoculated seedlings (28 and 21%, respectively).

DISCUSSION

Our results demonstrated that attractiveness of geranium seedlings to larval and adult female *B. impatiens* was greatly enhanced by presence of plant pathogenic Oomycetes (*Pythium* spp.). Seedlings infected/infested with various microbes, including Oomycetes, fungi, and bacteria, were also strong oviposition stimulants, as were pure-culture mycelial preparations of *Pythium* and two beneficial fungi (*T. harzianum* and *B. bassiana*). Inactivation of *Pythium* mycelia via freezing or desiccation rendered the mycelia unattractive as oviposition substrates. We did not conduct studies to identify the fundamental agent of attraction; however, the above-described findings with mycelia suggest that one or more chemical by-products of microbial metabolism and growth may be key.

Volatiles generated as a result of microbial activity have been implicated as oviposition stimulants for another agriculturally important dipteran, the onion maggot fly, *Hylemya antiqua* (7). In contrast to our finding, however, investigators found that these flies were not stimulated to oviposit by the test microbe (*Pseudomonas cepacia*) presented in pure culture; rather, oviposition stimulation was the result of an interaction between the microbe and the onion plant.

Table 2.9. Oviposition by fungus gnats on *Pythium aphanidermatum*- or *Trichoderma harzianum*-infected vs. non-infected (healthy) geranium seedlings grown in plug cells.

Assay – substrates tested	Number of replicate assays	Number of eggs [a]	Percentage of eggs [a]		MANOVA F-test
Pythium aphanidermatum-infected seedling	14	16.6 ± 4.2	71.9 ± 9.3	a	$F_{[1,13]} = 6.0$
V8 agar-inoculated seedling		7.2 ± 2.6	28.1 ± 9.3	b	P = 0.03
Trichoderma harzianum-inoculated seedling	10	18.1 ± 3.6	79.3 ± 9.1	a	$F_{[1,9]} = 11.1$
PDA-inoculated seedling		5.8 ± 2.8	20.7 ± 9.1	b	P = 0.009

[a] Mean percentage eggs (± standard error) deposited on each substrate in each assay (one female fungus gnat per replicate assay). Means within each assay followed by the same letter are not significantly different (MANOVA F-test; alpha = 0.05).

It was posited that the bacteria metabolize precursors in onion plants, converting them to volatile sulfide attractants/oviposition stimulants (18, 19).

Relatively few studies have investigated factors mediating substrate selection by sciarid fungus gnats. With respect to our results, a reasonable hypothesis would be that *B. impatiens* is attracted to or stimulated to oviposit by some non-specific factor associated with microbial activity. One obvious candidate for future investigations is the metabolic by-product CO_2. This compound is an important attractant of various insects, including other nematocerous diptera (e.g., foraging mosquitoes) (14). In addition, studies of *Lycoriella ingenua*, a common fungus gnat pest of mushroom cultures, revealed that oviposition on fungus-colonized litter increased when the litter was derived from plants grown in CO_2-enriched versus normal environments (10). Other studies, however, have indicated that this insect does not find all microbes equally attractive. Frouz and Nováková (9) reported that oviposition attractiveness of various fungi to this insect was highly variable, even among species of the same genus (in some cases, one species of a genus was highly attractive while another was completely unattractive). Additional detailed studies will be required to elucidate the precise mechanism(s) by which microbial activity elicits the observed responses from *Bradysia* fungus gnats.

We must recognize that oviposition site selection is a complex process, likely involving an interaction of physical, biological, and chemical factors. For some dipterans, oviposition attractants that result in oriented movement toward a source are the same (13, 21) as oviposition stimulants that elicit oviposition, while in other instances the chemicals may differ (2, 29). In this study, we did not isolate any compounds associated with infected/inoculated plants so it is not possible to discern whether the ovipositional attractant(s) and stimulant(s) are the same. Also, it is well known that visual cues play a pivotal role in oviposition for many insects (25),

and future studies should examine whether this sensory modality is important in fungus gnat oviposition.

Mechanisms underlying fungus gnat ovipositional preferences for beneficial microbes such as *Trichoderma* and *Beauveria* are also important to consider in greenhouse management. Microorganisms such as these are often applied to growing media to control soil-borne pathogens and insects (15, 35), and they may impact various aspects of the fungus gnat life cycle. However, demonstrated ovipositional preferences of sciarids for *Trichoderma* are quite variable in the literature. For example, Frouz and Nováková (9) found that some *Trichoderma* spp. were highly attractive to *L. ingenua* while others were completely unattractive. We observed attraction to *T. harzianum* pure cultures and inoculated seedlings, but Cloyd et al. (4) did not see attraction to *T. harzianum*-inoculated growing media. Potential for currently marketed beneficial microbes like *Trichoderma* and *Beauveria* to attract fungus gnats has implications for insect pest and disease management programs, and studies conducted under actual crop production conditions are needed.

Our study demonstrates that fungus gnats respond to a wide array of living microorganisms. This suggests that the commonly observed associations between fungus gnats and diseased plants are primarily the result of this attraction and will occur even in the absence of pathogen transmission by fungus gnats. Indeed, while fungus gnats are commonly associated with *Pythium* root rot disease outbreaks, we have found that these pests are not significant *Pythium* vectors, as the infectious propagules of these pathogens are produced primarily below ground (3). On the other hand, fungus gnat attraction to diseased /decaying plants obviously enhances contact between these insects and the readily acquired (and subsequently vectored) spores of many other pathogens produced in abundance on aerial plant parts (3, 5, 6, 8, 12, 16,

24). Although the microbial community to which fungus gnats are exposed likely changes with plant species, potting medium, and fertilization and irrigation practices, management strategies for these pests might include efforts to reduce unnecessary sources of microbial activity in the vicinity of potted plants (e.g., basic sanitation measures to remove plant debris) or to employ substrates supporting high microbial activity as trap baits. Such tactics will need to be balanced with providing crop plants with conditions that promote their defense and fitness. Overall, the findings of this study are important in enhancing our understanding of basic fungus gnat biology and interactions among fungus gnats, microorganisms, and crop plants.

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CHAPTER 3: Transstadial transmission of *Pythium* in *Bradysia impatiens* and lack of adult vectoring capacity

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ABSTRACT

Braun, S.E., Castrillo, L.A., Sanderson, J.P., Daughtrey, M.L., and Wraight, S.P. 2010. Transstadial transmission of *Pythium* in *Bradysia impatiens* and lack of adult vectoring capacity. Phytopathology 100: 1307-1314.

Fungus gnats have been shown to transmit a variety of plant-pathogenic fungi that produce aerial dispersal stages. However, few studies have examined potential interactions between fungus gnats and oomycetes, including *Pythium* spp. A series of laboratory experiments were conducted to determine whether fungus gnat adults are vectors of several common greenhouse *Pythium* spp., including *Pythium aphanidermatum*, *P. irregulare*, and *P. ultimum*. An additional objective was to determine whether *P. aphanidermatum* can be maintained transstadially in the gut of a fungus gnat larva through the pupal stadium to be transmitted by the subsequent adult. Adult fungus gnats did not pick up infectious *Pythium* propagules from diseased plants and transmit them to healthy plants in any experiment. Species-specific primers and a probe for real-time polymerase chain reaction were developed to detect the presence of *P. aphanidermatum* DNA in fungus gnat tissue samples. *P. aphanidermatum* DNA was detectable in the larval and pupal stages; however,

none was detected in adult fungus gnats. These results are in agreement with previous studies that have suggested that adult fungus gnats are unlikely vectors of *Pythium* spp.

INTRODUCTION

Fungus gnats of the genus *Bradysia* (Diptera: Sciaridae) are ubiquitous pests in greenhouse crop production systems (12). The larval stages of these insects inhabit the soil where they feed primarily on decaying plant materials and associated fungi (17, 27); however, in the absence of preferred foods, they will attack live, healthy plants. Damage is often limited and inconsequential and sometimes potentially beneficial (1) but large (late-instar) larvae may sever the primary roots or bore into the soft stems of young seedlings or cuttings with lethal effects. The short-lived adults of these insects do not cause any direct plant damage; they are generally described as either aphagous or as feeding on nectar or other liquids (17, 27). Steffan (27) described them as feeding "primarily on organic ooze." Because of their association with decaying vegetation, fungus gnats are commonly found to coexist with diseased plants, and their role as vectors of plant pathogens has been under investigation for many years. Studies have revealed that adult fungus gnats can acquire and mechanically transport the aerial spores of various plant pathogens. This has been most commonly demonstrated for fungal conidia, including those of *Fusarium*, *Thielaviopsis*, and *Verticillium* spp. (5–7, 10, 11, 16).

Although there is ample evidence that fungus gnats can vector aerial-spore-producing pathogens, a significant role in the transmission of pathogens that do not generate large numbers of such readily transmissible propagules has not been demonstrated. A large number of important pathogens of this type comprise the genus *Pythium* in the class Oomycetes (29). These fungus-like microbes (commonly called water molds) do not produce wind-disseminated

propagules; dispersal is via active movement of zoospores or passive transport in water, soil, or plant materials. As soil-borne pathogens, *Pythium* spp. are responsible for root and crown rots of mature plants and damping-off of seedlings. Persistence in the soil is by saprophytic growth and (primarily) by production of thick-walled sexual spores (oospores) in or on the infected tissues of their host plants. Their activity as root rot pathogens accounts for their frequent association with fungus gnats.

Studies over the past two decades have revealed that oospores of *Pythium* spp. are readily ingested by fungus gnat larvae, survive passage through the gastrointestinal tract, and, after being ejected in the frass, can germinate and infect plants under laboratory conditions (9, 14, 15). Larval fungus gnats, however, are soft-bodied, scotophilic inhabitants of soil and other substrates. Thus, their mobility is limited, and they are not likely to account for significant transmission of *Pythium* spp. between greenhouse facilities, between benches within a greenhouse, or even between separated pots on a greenhouse bench. *Pythium* spp. and other water molds are fully capable of persisting in soil as oospores and dispersing short distances through soil as zoospores or actively growing mycelia without reliance on insect vectors. Acquisition and transport of *Pythium* propagules by adult fungus gnats would be of much greater significance, yet this has been investigated in only a few studies. Favrin et al. (8) were unable to isolate *Pythium* spp. from adult fungus gnats collected near diseased plants in British Columbia greenhouses. Jarvis et al. (15) reported from direct microscopic observations that *Pythium aphanidermatum* (Edson) Fitzp. oospores ingested by *Bradysia impatiens* (Johannsen) larvae persisted in the body and were identifiable in the digestive tracts of the pupal and adult stages. However, relatively few oospores were observed in pupae and very few in the adults, and the oospores from neither of these life stages were found viable. Furthermore, adult fungus gnats did not ingest *P.*

aphanidermatum oospores suspended in sugar water or externally transmit *P. aphanidermatum* CFU from petri dishes with active cultures to dishes with a sterile agar substrate. These findings led Jarvis et al. (15) to conclude that "adults probably play only a minor role" in dissemination of *P. aphanidermatum*.

Despite this evidence of low potential for long-distance dissemination of *Pythium* spp. by fungus gnats, the perception persists that these insects are important vectors of these pathogens (14, 23). The primary objective of the current study was to further evaluate the *Pythium* spp.-vectoring potential of adult fungus gnats by assessing the capacity of *B. impatiens* adults to acquire infectious propagules of *P. aphanidermatum*, *P. ultimum* Trow, or *P. irregulare* Buisman from diseased plants and transmit them to healthy plants (geranium seedlings) under laboratory conditions highly favorable for infection. An additional objective was to develop and apply a molecular probe (real-time polymerase chain reaction [PCR]) to detect and quantify transstadial transmission of *P. aphanidermatum* in *B. impatiens*.

MATERIALS AND METHODS

Insect rearing

A laboratory colony of *B. impatiens* was established with adults collected from a greenhouse on the Cornell University campus. Adults and larvae were routinely reared in plastic containers (9.8 cm in diameter x 6.2 cm in depth) (Pioneer Plastics, Dixon, KY) with lids bearing holes (5.5 cm in diameter) covered with nylon mesh (95 μm) for ventilation. Fifty fungus gnat adults (predominately females) were placed into each container for oviposition and provided with ≈40 g of growing medium (Premier Pro-Mix BX, Quakertown, PA) mixed with ≈10 g (dry weight) of ground pinto beans (*Phaseolus vulgaris* L.) and saturated with tap water. Colony containers

were maintained at 27 ± 1ºC and 14 h of light and 10 h of darkness (14:10 L:D) for immature development and subsequent adult emergence.

To obtain an even-aged cohort of fourth-instar fungus gnat larvae for use in bioassays, ≈200 adult fungus gnats were collected from colony containers and released into a 45-by-45-by-45.5-cm cage in the laboratory at ambient temperature (≈24ºC) and allowed to oviposit on 90-by-15-mm petri dishes (Becton Dickinson Labware, Franklin Lakes, NJ) that contained saturated cotton covered with a piece of black filter paper spread with a thin layer (≈1.6 g) of ground pinto beans. After 24 h, petri dishes were removed from cages and the fungus gnat cultures maintained at 27 ± 1ºC until the larvae completed development to the fourth instar. Additional water and pinto beans were added to the dishes as needed to provide adequate food and water. Fourth-instar larvae were readily identified on the surface of the filter paper and transferred to bioassay containers using a fine brush.

Plant propagation

Geranium seed (*Pelargonium* x *hortorum* L.H. Bailey 'Orbit White'; Goldsmith Seeds) were surface sterilized in a 0.5% sodium hypochlorite solution and germinated individually in tight-lid petri dishes (47 by 10 mm) lined with one disk of filter paper (42.5 mm in diameter) saturated with 400 µl of sterile distilled water (SDH_2O). The seeds were incubated in darkness for 3 days at room temperature. The seedlings were then maintained at 27 ± 1ºC and 14:10 L:D and watered daily (with 200 µl of SDH_2O) until used in bioassays. The seedlings were oriented, for the most part, horizontally in the petri dishes (lying on the filter paper).

At the initiation of bioassays, seedlings were 8 days old (days post-seed hydration), ≈5 to 6 cm in length, and consisted of a short stem terminating in two cotyledons and an unbranched root with dense root hairs.

Pathogen maintenance and oospore production

All microbes used in the study are listed in Table 3.1. Three species of *Pythium* commonly affecting greenhouse crops (13) were selected for study. *P. aphanidermatum* strains Pa-58 and K-13, *P. ultimum* strain P4, and *P. irregulare* strain Par 1, were grown on V8 juice agar in 60-by-15-mm petri plates maintained in the dark at 27 ± 1ºC (recipe for 24 plates comprised 100 ml of V8 juice, 400 ml of H_20, 1.5 g of $CaCO_3$, and 10 g of Bacto Agar). One-week-old cultures of the various *Pythium* spp. were used in all experiments. Every 2 weeks, the pathogens were reisolated from 11-day-old geranium seedlings that had been inoculated 3 days earlier by placing a 6-mm-diameter agar disk (from 1-week-old culture) of *Pythium* on the root tip. Each infected seedling was placed in a 90-by-15-mm petri dish and covered with molten water agar (2%) supplemented with 1 ml of rifampicin, 1 ml of penicillin G, and 2.5 ml of kanamycin per liter of SDH_2O (WARP). *Pythium* hyphae that grew rapidly through the agar were excised from the surface of the medium and transferred to a fresh WARP plate. Additional strains and species of *Pythium* obtained for testing real-time PCR assay specificity were maintained on half-strength corn meal agar plates (Difco Laboratories, Detroit, MI) incubated in the dark at 25 ± 1°C (8.5 g of CMA per liter of SDH_2O).

Thielaviopsis basicola (Berk. & Broome) Ferraris cultures were grown on potato dextrose agar (MP Biomedicals, Solon, OH) in 60-mm-diameter petri dishes and maintained in the dark at 24 ± 1ºC. One-week-old cultures of *T. basicola* were used in all experiments. Every 2 weeks the

Table 3.1. List of microbes used in this study.

Species/strain	Host/Substrate	Year	Origin
Pythium aphanidermatum			
B-2	*Euphorbia pulcherrima*	2008	Pennsylvania
GDF2	*E. pulcherrima*	2007	Connecticut
GSM-1	*Pelargonium*	2006	Pennsylvania
H6-1	*Chrysanthemum*	2008	New York
Holl-2	*E. pulcherrima*	2007	New York
Jo-5	*Chrysanthemum*	2007	New York
K-13	*Chrysanthemum*	2007	New York
K-17	*Chrysanthemum*	2008	New York
Lab-14	*Chrysanthemum*	2007	New York
Lab-18	*Chrysanthemum*	2008	New York
P-52	Soil	2001	New York
Pa-58	*Gypsophila paniculata*	1999	Israel
Par-2	*Chrysanthemum*	2005	New York
P. acanthicum VB-37	Soil	2001	New York
P. angustatum TG-16	Soil	2000	New York
P. carolinianum NH-66	*E. pulcherrima*	2000	New Hampshire
P. dissotocum Bb-08-48	Soil	2008	New York
P. elongatum T-1	mix on fabric or floor	2000	New York
P. graminicola L-59	Soil	2001	New York
P. irregulare			
Par-1	*Pelargonium*	2005	New York
13-7	*Pelargonium*	1997	Michigan
13-10	*Pelargonium*	1997	Michigan
13-29	*Pelargonium*	1997	Florida
13-30	*Pelargonium*	1997	Florida
13-57	*Pelargonium*	1997	Florida
13-59	*Pelargonium*	1997	Florida
P. oligandrum DT-1	*Cupressus* × *leylandii*	2007	New York
P. plurisporium Wb-3	Water or irrigation system	2007	New York
P. splendens NF-3	*Acer*	2007	New York
P. sylvaticum BB-08-43	Soil	2008	New York
P. torulosum Pt-1	*Impatiens*	2001	New York
P. ultimum P4	*Phaseolus vulgaris*	1978	New York
P. vexans T-38	floor dirt	2000	New York
Thielaviopsis basicola	*Calibrachoa*	2007	New York

pathogen was reisolated from 18-day-old geranium seedlings that had been inoculated 10 days earlier as described above for the *Pythium* spp.

Oospores of *P. aphanidermatum* were produced in 90-by-15-mm petri dishes in a defined liquid medium composed of 3 mg of K_2HPO_4, 1.6 mg of KH_2PO_4, 0.17 mg of thiamine, 5 mg of $(NH_4)_2SO_4$, 1.1 mg of $CaCl_2$, 1.3 mg of asparagine, 0.18 g of d-glucose, and 1 g of lecithin per liter of SDH_2O (25) and maintained in the dark at 27 ± 1ºC for 12 days. Medium was dispensed in 20-ml aliquots into the petri dishes, and each dish was inoculated with a 6-mm-diameter agar disk (from 1-week-old culture) of *P. aphanidermatum* grown on V8 agar. Oospores were isolated from culture dishes by using a sterile scalpel to cut mycelium away from the agar inoculum disk. The resulting mat of *P. aphanidermatum* mycelium was placed in a sterile 50-ml centrifuge tube with 5 ml of SDH_2O and ≈1.15 g sterile 2-mm-diameter glass beads. Tubes were shaken for 10 min on a wrist-action shaker set at 6.7 oscillations/s (Model BT, Burrell Scientific, Pittsburgh, PA), and each *Pythium* suspension was poured through a sterile 53-µm sieve into a sterile 50-ml centrifuge tube. Samples were stored at -20°C until use.

Acquisition and retention of P.* aphanidermatum *by adult fungus gnats

A total of 100 assay replicates were conducted to determine whether adult fungus gnats are capable of acquiring and retaining *P. aphanidermatum* from culture dishes. Two 2-day-old female fungus gnats (from pupae surface sterilized in a 0.5% sodium hypochlorite solution) were used in each assay. Prior to the initiation of an assay, the vials containing fungus gnats were chilled in a refrigerator (4ºC) for 10 min to temporarily immobilize them during treatment.

Two treatments were included in each assay. In the first, one fungus gnat was grasped by the wings with fine forceps and dragged around the circumference of a *P. aphanidermatum*

culture on V8 agar. The inoculated fungus gnat was then transferred to a 60-mm-diameter petri dish and submerged in molten WARP that had been cooled to 45ºC. In the second treatment, a similarly dragged and inoculated fungus gnat was transferred to a dish containing solidified WARP. The plates were examined after 24 and 48 h for hyphal growth. If growth was evident after 2 days, the microbes were reisolated and grown on V8 agar to morphologically confirm the presence of *P. aphanidermatum*.

Transmission study with plants in plastic dishes

In total, 11 assays with *P. aphanidermatum* and 12 assays each with *P. ultimum* and *P. irregulare* were conducted to assess the capacity of adult fungus gnats to acquire and transmit *Pythium* spp. to healthy geranium seedlings. Twelve assays were conducted using *T. basicola* as a positive control because adult fungus gnats have previously been shown to transmit this pathogen (5). Batches of 8-day-old geranium seedlings were grown on filter paper and half of the seedlings in each batch were inoculated with agar plugs from *P. aphanidermatum*, *P. ultimum*, *P. irregulare*, or *T. basicola* cultures as previously described. All seedlings were maintained at 27 ± 1ºC and 14:10 L:D and watered daily (with 200 µl of SDH_2O) until used in bioassays. Seedlings used in *Pythium* sp. bioassays were 11 days old and those used in *T. basicola* bioassays were 15 days old at the start of the assay; by this time, all inoculated seedlings were patently infected or dead and exhibited external growth of *Pythium* hyphae or *Thielaviopsis* conidiation. Ten 2-day-old fungus gnats (five males and five females) were used in each assay. Assays were conducted in plastic containers (9.8 cm in diameter by 6.2 cm in depth) with tight-fitting, unventilated lids. The bottom of each container was lined with a piece of filter paper (90 mm in diameter) saturated with 1.5 ml of SDH_2O. Four small (35 mm in

diameter) petri dishes, each containing 5 ml of water agar covered with a 25-mm-diameter piece of filter paper saturated with 100 μl of SDH_2O, were placed in the bottom of each assay container.

Three treatments were included in each assay. For the first treatment, two healthy (noninoculated) seedlings were placed diagonally across from one another in the small petri dishes, and two infected seedlings were placed in the remaining two dishes (Fig. 3.1). Ten fungus gnats (five female and five male) were then released in the assay container. The second treatment was the same as the first except no fungus gnats were added. For the third treatment, four noninoculated plants plus 10 fungus gnats (5 female and 5 male) were added to the assay container. In all treatments, noninoculated plants were examined for disease symptoms after incubation for 7 days under the above-described environmental conditions. Adult fungus gnats are short lived; they survived and were active in the containers for ≈2 days.

In the case of *P. ultimum*, assays were conducted in larger plastic containers (15.0 cm in diameter by 2.5 cm in depth) (BD Falcon, Franklin Lakes, NJ) lined with a 125-mm-diameter piece of filter paper saturated with 2 ml of SDH_2O; matching containers were used as lids. After 2 days in the presence of fungus gnats, each pair of noninoculated plants on the small petri dishes from the three treatments was transferred to a clean 9.8-by-6.2-cm assay chamber. It was necessary at this time to separate the noninoculated plants from the diseased plants because preliminary assays had revealed that hyphae of *P. ultimum* were capable of rapid growth from dish to dish (across the moist filter paper lining the bottom of the chamber).

Transmission study with seedlings on plug mix

In total, 12 assays were conducted to further assess the capacity of adult fungus gnats to acquire and transmit *P. aphanidermatum* to healthy geranium seedlings. Infected seedlings were obtained as previously described. In this experiment, infected or noninfected seedlings were laid directly on the surface of the potting medium in expectation that provision of an attractive oviposition site might enhance the contact between the fungus gnats and the seedlings. Assays were conducted in the 9.8-cm-diameter containers filled with ≈40 g of Redi-Earth Plug and Seedling Mix (Sun Gro Distribution Inc., Bellevue, WA) saturated with SDH_2O.

Two treatments were included in each assay. In all, thirty-five 11-day-old geranium seedlings showing root rot characteristic of *P. aphanidermatum* infection were placed on top of the plug mix in one container, and 35 11-day-old noninoculated geranium seedlings were placed on top of the plug mix in a second container (seedlings were distributed as evenly as possible) (Fig. 3.1). Fifty 2-day-old female fungus gnats were then released into each container. After 24 h, 20 fungus gnats from each treatment were collected with forceps and transferred to new assay containers, with 35 healthy seedlings also on plug mix, as described. These plants were examined for disease symptoms after 7 days. An additional 10 fungus gnats from each treatment were removed from the original assay containers, placed in 60-mm-diamater petri dishes, and covered with molten WARP. The plates were examined after 24 and 48 h for *P. aphanidermatum* growth.

Transstadial transmission

The intent of this experiment was to determine whether *P. aphanidermatum* oospores ingested during the ultimate larval instar persist in the fungus gnat body through pupation and adult eclosion. Using a fine brush, groups of 25 early-fourth-instar larvae were transferred from

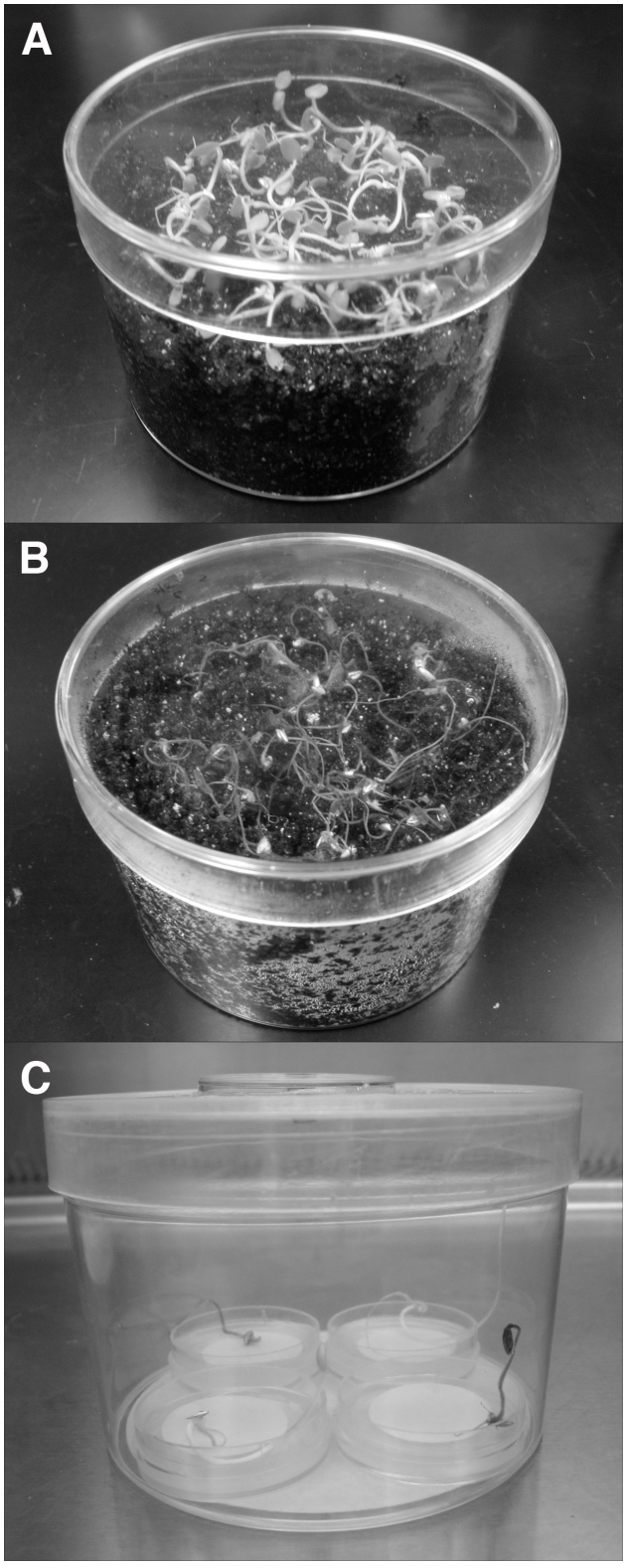

Figure 3.1. A, Noninoculated geranium seedlings in transmission study with seedlings on plug mix. **B**, Geranium seedlings infected with *Pythium aphanidermatum* in transmission study with seedlings on plug mix. **C**, Set-up for transmission study with plants in plastic dishes.

rearing dishes to each of 21 petri dishes containing 2% water agar. Larvae were maintained in the dark at 27 ± 1ºC and allowed to feed on the water agar for 6 h to clear their guts. They were then transferred onto sterile filter paper saturated with SDH_2O and allowed to move on this substrate for 10 min to dislodge surface contaminants. At this point, 3 or 4 larvae were selected from each of three randomly sampled plates and placed into sterile 1.5-ml microcentrifuge tubes (1 larva/tube, total of 10 larvae). All specimens were then frozen at ≈-20°C until analyzed. Each group of larvae from each of the remaining 18 dishes was transferred to a V8 agar plate containing a *P. aphanidermatum* culture from which the aerial mycelium had been removed using a sterile scalpel. This plate feeding method was used because fungus gnats could not be induced to feed on purified preparations of oospores produced in the defined liquid medium. After 15 h, a sample of three or four larvae was taken from each of three randomly selected plates and placed on sterile filter paper saturated with SDH_2O to remove surface contaminants. Each larva was then put into a sterile 1.5-ml microcentrifuge tube and frozen. This sampling was repeated from an additional three randomly selected plates at 48 h from the start of the experiment. Pupae were similarly sampled from all culture plates within 24 h after pupation. Additional pupae were transferred to dishes lined with moist filter paper for adult emergence; adults were collected within 1 h of eclosion and immediately frozen.

The entire experiment was conducted three times with *P. aphanidermatum* strain Pa-58, providing grand totals of 26 to 33 insects of each age group. Most adult fungus gnats (28 of 33) were collected from the third experimental replicate after modifications were made to the handling protocol (pupae removed too quickly from their pupation sites in the culture plates suffered high rates of mortality). To account for any possible strain variation in transstadial transmission, the above-described experiment was also conducted once with *P. aphanidermatum*

strain K-13, providing totals of 15 pupae and 25 adults for molecular analysis. Eight meconial deposits were examined microscopically (×400) for the presence of oospores immediately after adult emergence in plastic dishes.

DNA extraction from oomycetes and fungus gnats

For DNA extraction from *Pythium* spp. for PCR assays, a 0.5-cm agar plug from the growing edge of each strain was inoculated into 8 ml of potato dextrose broth in a 60-by-15-mm petri dish and incubated in the dark at 25°C for 4 to 7 days. Mycelium (≈100 mg, wet weight) was collected in a 2-ml bead beating tube (Sarstedt, Newton, NC), washed with sterile distilled water, and spun for 10 min at 14,000 rpm, and the pellet used immediately for DNA extraction. Zirconia/silica beads (0.5 g) and 400 μl of AP1 lysing buffer with 4 μl RNase (Qiagen DNeasy Plant Mini Kit; Qiagen, Valencia, CA) were added to each tube and the samples were homogenized for 30 s at 4,200 rpm in a Mini Bead Beater (BioSpec Products, Bartlesville, OK). Subsequent DNA extraction steps proceeded as recommended by the manufacturer (Qiagen). DNA was eluted in 100 μl of Tris (pH 8.0), quantified using a spectrophotometer (Eppendorf, Westbury, NY), and stored at -20°C until use.

To equate DNA quantity with oospore numbers, oospores of *P. aphanidermatum* strain Pa-58 were suspended in sterile distilled water at a stock concentration of 1.68×10^5 per 100 μl quantified via hemacytometer counts. Expected concentrations of 8.4×10^4, 1.68×10^4, 8.4×10^3, 1.68×10^3, 840, and 168 oospores per 100 μl were subsequently prepared from a series of alternating five- and twofold dilutions of the stock suspension. A 100-μl sample of each oospore suspension was then spun for 10 min at 14,000 rpm and the pellet re-suspended in 400 μl of AP1

lysing buffer. Samples were ultimately processed using the same DNA extraction protocol described for mycelia.

For DNA extraction from fungus gnats, individual specimens (larvae, pupae, and adults) were surface sterilized by immersion in 70% ethanol, washed twice with sterile distilled water, dried on autoclaved filter paper, and transferred to 1.5-ml sterile microcentrifuge tubes. Each specimen was triturated in 50 µl of AP1 lysing buffer (Qiagen) using a sterile 1.5-ml Kontes pellet pestle (Kimble Chase, Vineland, NJ). Another 350 µl of lysing buffer was added and DNA extraction proceeded as reported above. The grinding step was added to break up the insect sample and release its gut content. DNA was eluted in 50 µl of Tris pH 8.0 and stored at -20°C until use.

PCR primers and Taqman probe

Real-time PCR primers and probe were designed using Beacon Designer software (version 6.1; Premier Biosoft International, Palo Alto, CA), based on *P. aphanidermatum* ITS complete sequence and partial sequence of the 5.8S rRNA gene (Accession AF310331). This sequence was amplified with *P. aphanidermatum*-specific primers Pa1 and ITS2 (30). We also tested the specificity of this primer pair by running standard PCR assays against several more *P. aphanidermatum* and other *Pythium* spp. strains using conditions reported by Wang et al. (30). Several real-time PCR primer pairs were developed and again tested for specificity against a set of *P. aphanidermatum* and *Pythium* spp. strains by standard PCR. PCR reaction mixtures (25 µl) contained 1 × PCR buffer with 1.5 mM $MgCl_2$; 200 µM each of dATP, dCTP, dGTP, and dTTP; 0.5µM of each primer, 1.0 U of Taq polymerase (Qiagen); and 20 ng of template DNA. PCR amplification was performed in a PTC-200 thermal cycler (MJ Research, Waltham, MA)

programmed for initial denaturation at 94°C for 4 min; 30 cycles of 94°C for 1 min, 56 to 66°C or 62°C for 1 min; and extension at 72°C for 1 min. PCR products were visualized on 1% agarose gels stained with ethidium bromide. Assays were repeated at least twice for each strain. Additional specificity assays were conducted using real-time PCR against a subset of the strains and species initially tested. The selected primers were Pa4F (5'-GAACCGTTGAAATCATGTTCTGTG) and Pa1R (5'-TACATCGGCAGACTACAATTAAGC), which amplified a 81-bp product. This primer pair was specific to *P. aphanidermatum* and also showed the lowest calculated threshold (Ct) values. The Ct value is the cycle at which a given sample reaches a point of fluorescent intensity above background, which can be correlated to the starting concentration of target DNA. The probe PaTq (5'- CCCACCTTCGTTCAGCCCTCCCG) was labeled at the 5' end with fluorescent FAM reporter dye and at the 3' end was TAMRA quencher dye (Integrated DNA Technologies, Coralville, IA).

Real-time PCR

Real-time PCR assays were conducted using the iCycler iQ5 real-time PCR detection system (BioRad Laboratories, Foster, CA). Each reaction mixture (25-µl final volume) contained 1× iQ Supermix (BioRad Laboratories), 0.5 µM each primer, 0.125 µM probe, and 2 µl of each of the standard or sample DNA. Thermal cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and a single-step annealing and extension at 60°C for 1 min. Each PCR assay included a standard curve of 6 to 7 series 10-fold dilution from 40 ng to 0.4 pg or 10 ng to 0.001 pg of Pa-58 DNA (or K-13 DNA for assays with that strain) for determining the starting concentrations of unknowns and a non-template control.

Assays were performed on duplicate samples and at least two independent assays were performed for each experiment.

Statistical analyses

Statistical tests were conducted using the JMP software version 7.0 (26). A linear regression analysis (with intercept constrained to zero) was conducted of *P. aphanidermatum* DNA detected versus oospore number. Analysis of variance (ANOVA) followed by the Tukey-Kramer test for means comparisons were used to examine differences in amounts of *P. aphanidermatum* DNA in insect samples. The log (X + 1) transformation was applied prior to ANOVA to normalize the distributions and equalize the treatment variances.

RESULTS

Acquisition and retention of P. aphanidermatum *by adult fungus gnats*

In the case of the 45ºC water-agar treatment, *P. aphanidermatum* colonies grew on 11% of the plates. *P. aphanidermatum* colonies grew on only 1% of the plates in the solidified water-agar treatment.

Transmission study with plants in plastic dishes

For all three treatments with the pathogens *P. aphanidermatum*, *P. ultimum*, and *P. irregulare*, the seedlings remained healthy and free of disease symptoms after 7 days. With *T. basicola*, the positive control, 10 of the 12 noninoculated (healthy) seedlings became infected in the dishes containing 2 diseased seedlings, 2 healthy seedlings, and 10 fungus gnats. Only 1 of 12 healthy

seedlings became infected with *T. basicola* in the dishes without fungus gnats. No healthy seedlings became infected in the untreated controls (Table 3.2).

Transmission study with seedlings on plug mix

In both treatments (35 infected geranium plants with 50 fungus gnats or 35 noninoculated plants with 50 fungus gnats), plants remained healthy and free of disease symptoms after 7 days (Table 3.2). Also, no *P. aphanidermatum* growth was observed on the water-agar plates onto which fungus gnats that had been exposed to diseased plants were released.

***Correlation of P. aphanidermatum* DNA with oospore number**

The relationship between estimated amounts of DNA and numbers of oospores was described by the equation 0.0004 × number of oospores (Fig. 3.2). However, because larvae ingested a mix of mycelia and oospores in this study, we were not able to use this relationship to determine numbers of oospores ingested (see Discussion).

Transstadial transmission

For strain Pa-58, no evidence of *P. aphanidermatum* was detected in larvae that fed on water agar ($n = 30$). However, larvae placed on *P. aphanidermatum* cultures overnight ($n = 30$) contained an average of 24.52 ± 8.68 picograms of *P. aphanidermatum* per insect, with 100% of the samples containing some level of *P. aphanidermatum* (Table 3.3). Larvae placed on *P. aphanidermatum* cultures for 48 h had less *P. aphanidermatum* per insect, with 80% of the samples containing some level of *P. aphanidermatum*. Insects retained the pathogen until pupation, with 92% of the samples containing some level of *P. aphanidermatum*. No *P.*

Table 3.2. Fungus-gnat-mediated transmission of *Thielaviopsis basicola* and lack of transmission of three *Pythium* spp. in laboratory assays with geranium seedlings.

	Percent transmission[a]			
Treatment	*T. basicola*	*Pythium aphanidermatum*	*P. ultimum*	*P. irregulare*
Seedlings on filter paper[b]				
DS + HS with FG	83 (10/12)	0 (0/11)	0 (0/12)	0 (0/12)
DS + HS without FG	8 (1/12)	0 (0/11)	0 (0/12)	0 (0/12)
Control (HS with FG)	0 (0/12)	0 (0/11)	0 (0/12)	0 (0/12)
Seedlings on peat-based plug mix[c]				
FG from DS transferred to HS	–	0 (0/12)	–	–
Control (FG from HS transferred to HS)	–	0 (0/12)	–	–

[a] Percentage of assay chambers (replicates) with disease transmission to the initially healthy plants. Numbers in parentheses indicate number of experimental replicates (assay chambers) in which transmission was observed divided by the total number of replicates.
[b] Two diseased seedlings + two healthy seedlings (DS + HS) or four HS placed together (but not contacting one another) in an assay chamber with 10 adult fungus gnats (FG) or without FG.
[c] Twenty FG exposed to 35 DS or 35 HS (control) for 24 h and then transferred to a chamber with 35 HS.

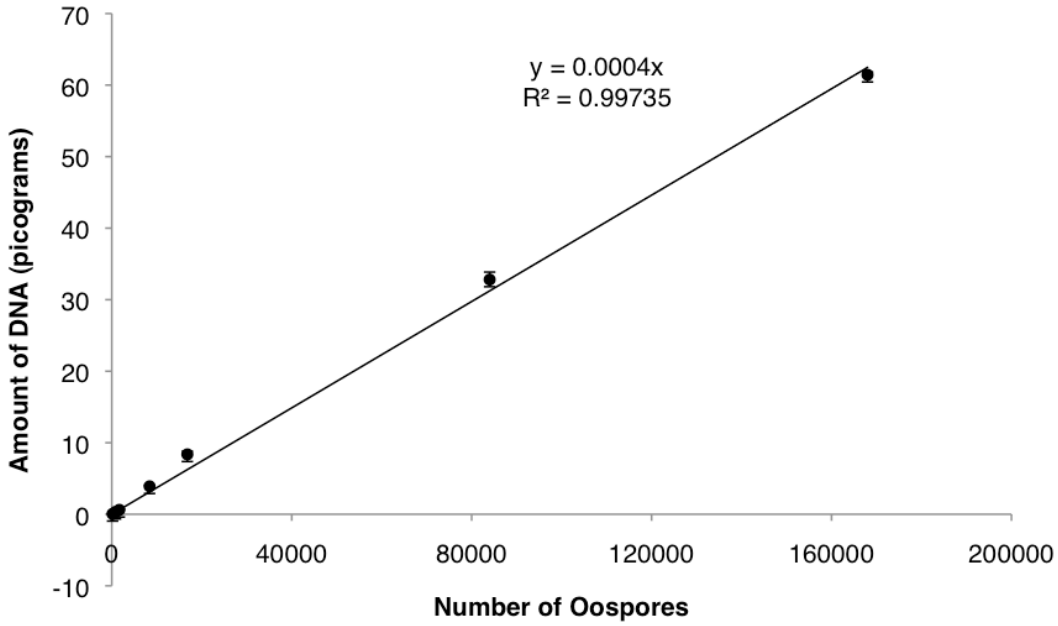

Figure 3.2. Relationship between number of *Pythium aphanidermatum* strain Pa-58 oospores and amount of DNA in picograms.

Table 3.3. Mean picograms of *Pythium aphanidermatum* in insect samples detected by real-time polymerase chain reaction

Treatment	No. of specimens	*P. aphanidermatum*/specimen (pg) (range)[a]
Strain Pa-58		
Larvae fed agar	30	0
Larvae fed *Pythium* for 15 h	30	24.52 ± 8.68 a (0.02-215)
Larvae fed *Pythium* for 48 h	30	3.01 ± 1.09 b (0-22.62)
Pupae	26	5.24 ± 1.33 b (0-22.25)
Adults	33	0
Strain K-13		
Pupae	15	6.01 ± 1.31 (0-10.14)
Adults	25	0

[a]Mean picograms of *P. aphanidermatum*/insect specimen (range of values). Means followed by the same letter are not significantly different (Tukey-Kramer test; $\alpha = 0.05$).

P. aphanidermatum was detected in adults (n = 33), suggesting that they did not retain any *P. aphanidermatum* from larval feeding (Table 3.3).

Similar results were observed with *P. aphanidermatum* strain K-13. Oospores were readily observed in larvae that fed on *P. aphanidermatum* K-13 culture plates (Fig. 3.3), and 93% of the pupal samples contained some level of *P. aphanidermatum*. The molecular probe detected no *P. aphanidermatum* in the adults (n = 25) (Table 3.3), and we observed no microscopic particles resembling *Pythium* oospores in the excreted meconia.

DISCUSSION

Pythium spp. are responsible for a broad range of root rot, crown rot, and cutting diseases in floricultural crop production (21). Primary sources of *Pythium* spp. in the greenhouse include diseased plants (especially plants with subclinical infections), contaminated potting media, irrigation water, and soil adhering to cultivation equipment and grower's clothing. In addition, growers and scientists have long suspected that fungus gnats could be another significant source of inoculum in disease outbreaks. However, the results from our investigations, in conjunction with the observations of Favrin et al. (8) and the findings of Jarvis et al. (15), strongly indicate that this is not the case -- that fungus gnats, in fact, have a very low *Pythium* spp.-vectoring potential.

Only 11% of the fungus gnats that were dragged through *P. aphanidermatum* cultures produced *Pythium* colonies when immersed in molten agar and just 1% when placed on solidified agar. This suggests that most of the acquired inoculum consisted of fragmented hyphae that were either never viable or rapidly lost viability. CFU likely consisted of large masses of hyphae (possibly including oogonia and oospores) deposited on the legs or bodies of

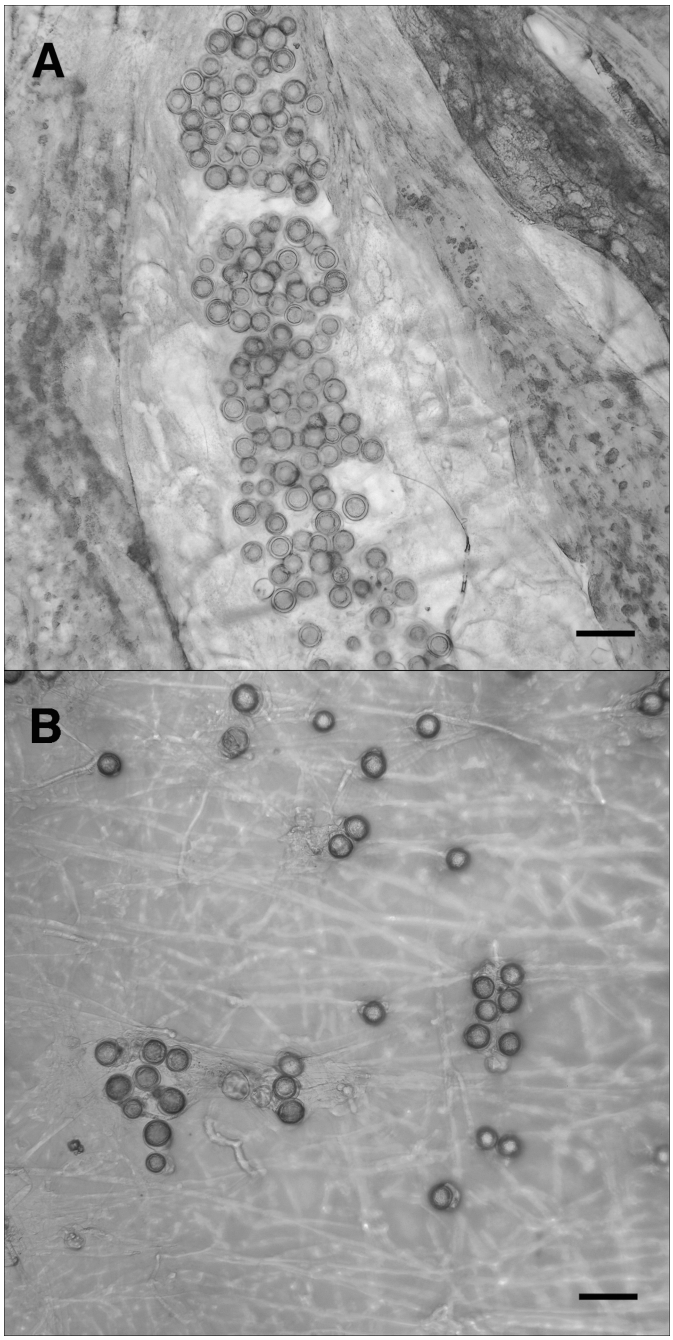

Figure 3.3. A, Oospores of *Pythium aphanidermatum* strain K-13 in the digestive tract of a fungus gnat larva. **B**, Surface of *P. aphanidermatum* strain K-13 culture plate where fungus gnat larvae were placed to feed. Scale bars = 50 μm.

the insects by the force of the artificial manipulation. In contrast, adult fungus gnats permitted to move freely (naturally) on the diseased seedlings or *Pythium* spp.-contaminated plug mix did not pick up and transmit an infectious dose or CFU of inoculum in any of our tests. In our cultures of *P. aphanidermatum*, *P. ultimum*, and *P. irregulare*, few oospores and no sporangia were observed on aerial hyphae that would have been subject to collection by the fungus gnats. Oospore production was confined primarily to the agar matrix.

In their natural habitats, the sexual and asexual reproductive structures of *Pythium* spp. are produced primarily belowground, inaccessible to adult fungus gnats. The mycelia of these microbes are made up of fine, coenocytic filaments that are extremely vulnerable to desiccation (20) and represent an equally unlikely source of inoculum for fungus gnat transmission. Many species of *Pythium*, including *P. irregulare* and *P. ultimum* (but not *P. aphanidermatum*) produce thin-walled asexual spores commonly referred to as hyphal swellings (18, 28). Though potentially becoming airborne via soil dust or rain splash, these spores are not specifically adapted for wind dissemination like the caducous, desiccation-tolerant conidia of many fungi and, thus, are also unlikely to be picked up by insect vectors. Of the fungal plant pathogens known to be transmitted externally by adult fungus gnats, all produce abundant conidia aboveground on infected host tissue. In the case of *T. basicola*, used as a positive control in this study, these conidia were readily acquired and disseminated by adult fungus gnats.

We were unable to detect *P. aphanidermatum* DNA in adult fungus gnats using real-time PCR. This would appear to contradict the observations of Jarvis et al. (15), who reported finding *P. aphanidermatum* oospores in 1.7% of adult fungus gnats fed *Pythium* during the larval stage. Actual numbers of oospores observed in these adults were exceedingly low, however ($\leq$ 2/adult), and such low numbers are likely below the detection threshold of our PCR assay. The smallest

amount of DNA detected in our tests was 0.002 pg, which according to the estimate of 0.0004 pg/oospore would represent approximately five oospores. Positive visual identification of individual microbial spores in insect tissues is exceedingly difficult (it was this difficulty, in fact, that prompted us to develop a molecular probe), and we did not attempt examination of pupal or adult digestive tracts.

The large amounts of *P. aphanidermatum* DNA found in the actively feeding larvae in our experiments (mean: 25 pg/larva) translates to > 60,000 oospore equivalents. Ingestion of agar with oospores at the densities produced by the *P. aphanidermatum* isolates used in this study could not have produced such numbers (Fig. 3.3B), and we did not observe high concentrations of oospores in any larvae. To account for this discrepancy, it must be concluded that most of the detected DNA came from ingested hyphae. This could explain the precipitous drop in the amount of DNA found in larvae allowed to feed for 48 h (Table 3.3). Many of these larvae were nearing pupation by that time and had ceased feeding. During the prepupal or so-called wandering period, digestion continues (4), and much of the DNA, especially that ejected from ruptured hyphae, was probably subjected to rapid degradation. Even so, large amounts of DNA (>10,000 oospore equivalents) persisted into the pupal stage. It may be that a portion of the hyphal DNA was protected in some way, perhaps encapsulated in agar or within the walls of large hyphal fragments or hyphal aggregates (mycelia).

The lack of transstadial transmission of *Pythium* DNA or viable propagules reported herein and by Jarvis et al. (15) is not unexpected. Insects undergo profound structural and physiological changes (histolysis/histogenesis) during metamorphosis that present a considerable barrier to persistence of ingested materials. The insect foregut and hindgut linings (intima) are shed during each molt, and during the pupal stage of holometabolous insects, the midgut is

completely reformed around the larval gut, which is then assimilated for reuse. Following emergence from the pupal cuticle, waste from this and other processes is voided as a fecal fluid called the meconium (2). Many studies have reported a lack of transstadial transmission of plant-pathogenic viruses, bacteria, and fungi by insects (3, 19, 22, 24). El-Hamalawi (5) found that fungus gnat larvae that fed on either *Verticillium dahliae*, *Fusarium acuminatum*, or *T. basicola* developed into internally infested pupae. However, as in our study, emerging adults were void of fungal propagules. In one case where transstadial retention of a plant-pathogenic bacterium, *Serratia marcescens* Bizio, was observed, only 9 of 82 squash bugs that fed on *Serratia* as fifth-instar nymphs transmitted the bacterium to plants as adults (31). The process of metamorphosis also differs significantly between hemimetabolous insects such as squash bugs and holometabolous insects such as flies, and distinctive stages in their life cycles could explain the unique transstadial transmission potential of certain insect groups.

The findings of this study enhance our understanding of the association between fungus gnats and *Pythium* spp. in greenhouse floriculture. The molecular assay that we developed for species-specific detection and quantification of *P. aphanidermatum* will likely be useful to plant pathologists and entomologists for a variety of future studies. Although we have demonstrated that fungus gnats are unlikely vectors of *Pythium* spp., relaxing management strategies for fungus gnats is not currently warranted. Fungus gnats should still be considered pests in most cases because they are capable of directly wounding plants and transmitting several plant pathogenic conidial fungi (10, 12, 16). Future disease epidemiology studies with fungus gnats should focus on pathogen-vector-host specificity and the likelihood of transmission in various ecological settings.

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CHAPTER 4: Larval *Bradysia impatiens* vectoring potential of *Pythium* root rot pathogens

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ABSTRACT

A series of laboratory experiments were conducted to investigate the capacity of *Bradysia impatiens* (Johannsen) larvae to ingest propagules from two strains each of *Pythium aphanidermatum* (Edson) Fitzp. and *P. ultimum* Trow and transmit the pathogens to healthy geranium seedlings in petri dishes. The vectoring potential of larval fungus gnats in a potting mix system and the germination rate of *Pythium* spp. oospores and hyphal swellings before and after passage through the guts of larval fungus gnats were also examined. Assays revealed that the *Pythium*–vectoring potential of larval fungus gnats varies greatly by the density and nature of ingested propagules. Transmission rate was highest when larvae were placed on non-infected seedlings in petri dishes after feeding on *P. aphanidermatum* strain K-13, although the rate of transmission was much lower in potting mix. Larvae were less efficient at vectoring *P. ultimum* strain PSN-1, and no transmission was observed with *P. aphanidermatum* strain Pa58 or *P. ultimum* strain P4. Passage of *P. aphanidermatum* strain K-13 through larval guts significantly increased oospore germination rate, although decreased hyphal swelling germination was observed following larval gut passage for strains of *P. ultimum*. These results expand previous studies suggesting that larval fungus gnats may vector *Pythium* spp.

INTRODUCTION

Root-feeding insects influence crop production in a variety of settings, including agricultural fields, turf grass plots, and greenhouses (4, 6, 14, 21). In greenhouse crop-production systems, fungus gnats of the genus *Bradysia* (Diptera: Sciaridae) are ubiquitous pests. The larvae of these insects inhabit the soil or soilless potting media, where they feed primarily on decaying plant material and associated fungi (20), but larvae of several species, including the subject of the present study *Bradysia impatiens* (Johannsen), also consume root, stem, and occasionally leaf tissues of healthy plants, potentially causing significant damage, especially to seedlings and cuttings (31). Wounding by larvae may also have a variety of effects on plant susceptibility to disease. Some studies have shown that fungus gnat feeding damage can promote plant infection by pathogenic fungi (12, 22), whereas others have demonstrated no enhanced infection (11) or even potentially beneficial relationships in which feeding damage can lead to induced resistance to plant pathogens (3). Fungus gnats also commonly coexist with diseased plants and may interact with plant pathogens as disease vectors (14).

The external body surfaces of adult fungus gnats are readily contaminated with the aerial conidia of various plant pathogenic fungi, including those of *Fusarium*, *Thielaviopsis*, and *Verticillium* spp., and these propagules are readily transmitted to healthy plants (2, 7, 8, 9, 11, 13, 19). However, fungus gnat adults are less likely to mechanically vector other important plant pathogens such as *Pythium* spp. in the class Oomycetes because these pathogens do not generate large numbers of aerially-transmissible spores (2, 17). Oomycetes are normally dispersed via active movement of asexual zoospores or passive transport in water, soil, or plant materials. Their persistence in the soil is marked primarily by the production of thick-walled sexual spores known as oospores in or on infected host tissues (30). Adults of *B. impatiens* are generally

described as aphagous and thus are also unlikely to transmit pathogens internally (20). Jarvis et al. (17) were unable to induce adult *B. impatiens* to ingest *Pythium* oospores suspended in water or water with 1% glucose. It has also been demonstrated that *Pythium* propagules readily ingested by *B. impatiens* larvae are not transstadially transmitted to the adult gnats (2).

Though not passed to the adult stage, *Pythium* oospores and chlamydospore-like hyphal swellings have been shown to survive passage through the larval digestive tract and, upon excretion, infect plants grown in rockwool or petri dishes under laboratory conditions (10, 16, 17). To our knowledge, however, no studies have examined the vectoring potential of larval fungus gnats in a potting mix system or the influence of pathogen strain on transmission. The primary objective of the current study was to evaluate the capacity of *Bradysia impatiens* (Johannsen) larvae to ingest propagules from two strains each of *Pythium aphanidermatum* (Edson) Fitzp. and *P. ultimum* Trow and transmit the pathogens to healthy geranium seedlings under various conditions. Survival of oospores and hyphal swellings passed through the guts of larval fungus gnats was also quantified.

MATERIALS AND METHODS

Insect Rearing. A laboratory colony of *B. impatiens* was established with adults collected from a greenhouse on the Cornell University campus. Adults and larvae were routinely reared in plastic containers (9.8 cm diam. x 6.2 cm depth; Pioneer Plastics, Dixon, KY) with lids bearing holes (5.5 cm diam.) covered with nylon mesh (95 µm) for ventilation. Each container was provided with 40 g of growing medium (Premier Pro-Mix® BX, Quakertown, PA) mixed with 10 g (dry weight) of ground pinto beans (Goya® Foods, Inc., Secaucus, NJ). The mix was then saturated with tap water, and 50 fungus gnat adults (predominately females) were introduced for

egg production. Colony containers were maintained at 27 ± 1ºC and 14h:10h Light:Dark (L:D). In the following description of methods, these specific temperature/light conditions will be referred to as the "standard incubation conditions" or simply "standard conditions." New containers were established daily to provide a constant supply of all life stages. To obtain an even-aged cohort of fourth-instar fungus gnat larvae for use in bioassays, approximately 200 adult fungus gnats were collected from colony containers and released into a 45 cm x 45 cm x 45.5 cm cage in the laboratory at ambient temperature and allowed to oviposit on 90-mm-diam. Petri dishes (Becton Dickinson Labware, Franklin Lakes, NJ) that contained saturated cotton covered with a piece of black filter paper spread with a thin layer (approximately 1.6 g) of ground pinto beans (*Phaseolus vulgaris* L.). After 24 h, Petri dishes were removed from cages and the fungus gnat cultures maintained until the larvae completed development to the fourth-instar. Additional water and pinto beans were added to the dishes as needed. Fourth-instar larvae were readily identified on the surface of the filter paper and transferred to bioassay containers using a fine brush.

Plant Propagation. Geranium seeds (*Pelargonium* x *hortorum* L.H. Bailey 'Orbit White', Goldsmith Seeds®) were surface sterilized in a 0.5% sodium hypochlorite solution and germinated individually on top of a filter paper disk (42.5-mm diam.) saturated with 400 µl sterile distilled water (SDH_2O) in 47-mm diam. friction-lid Petri dishes (Fisher Scientific, Pittsburgh, PA, Cat. No. 09-720-501). The seeds were incubated in darkness for 3 days at room temperature. Seedlings were maintained under standard conditions and watered daily with 200 µl SDH_2O until used in bioassays. The seedlings were oriented, for the most part, horizontally in the Petri dishes (lying on the filter paper).

Seeds used in plug cell bioassays were planted in tap water-saturated peat-based Redi-earth Plug and Seedling Mix (Sun Gro Horticulture Canada Ltd., Vancouver, BC) within individual plug cells (ca. 14 ml volume) cut from 128-well plastic plug trays (Dillen Products/Myers Industries Inc., Middlefield, OH) and incubated in darkness at 23ºC until germination (4 days). Seedlings were maintained under standard conditions until used in bioassays.

Pathogen Maintenance. *Pythium aphanidermatum* strain Pa58 and *Pythium ultimum* strain P4 were obtained from a culture collection maintained at Cornell University, Ithaca, NY (E.B. Nelson lab), and *P. aphanidermatum* strain K-13 and *P. ultimum* strain PSN-1 were obtained from a collection maintained at the Long Island Horticultural Research and Extension Center (LIHREC), Riverhead, NY (M.L. Daughtrey lab). *P. aphanidermatum* cultures were grown in 60-mm-diam. Petri dishes on V8 juice agar (composed of 100 ml V8 juice, 400 ml H_20, 1.5 g $CaCO_3$, and 10 g Bacto^{TM} Agar) and maintained in the dark at 27 ± 1ºC. *P. ultimum* cultures were grown on half-strength corn meal agar (composed of 8.5 g corn meal agar per liter of SDH_2O) and maintained in the dark at 25 ± 1ºC. One-week-old *P. aphanidermatum* and ten-day-old *P. ultimum* cultures were used in all experiments and for inoculating plants for periodic reisolation of the pathogens. Every two weeks the pathogens were re-isolated from 11-day-old geranium seedlings that had been inoculated 3 days earlier by placing a 6-mm-diam. *Pythium*-colonized agar disk on the root tip. Each infected seedling was placed in a 90-mm-diam. Petri dish and covered with 2% molten water agar supplemented with the antibiotics rifampicin, penicillin G, and kanamycin (1.0, 1.0, and 2.5 ml/l SDH_2O, respectively and each 0.01 g/ml)

(WARP). *Pythium* hyphae that grew rapidly through the agar were excised from the surface of the medium and transferred to a fresh WARP plate.

Transmission study with seedlings in plastic dishes. Three laboratory bioassays (16-20 replicates/treatment) were conducted to determine whether *P. aphanidermatum* consumed by fungus gnat larvae could be transmitted to geranium seedlings in petri dishes. Using a fine brush, individual early-fourth-instar larvae were transferred from rearing dishes to each of 16-20 petri dishes containing 2% water agar. Larvae were maintained in the dark at 27 ± 1ºC and allowed to feed on the water agar for 6 h to clear their guts. Larvae were then transferred to V8 agar plates (1 larva/plate) containing a *P. aphanidermatum* culture of either strain K-13 or Pa58 from which the aerial mycelium had been removed using a sterile scalpel. After 15 h of feeding, larvae were removed from the culture plates and transferred individually onto sterile filter paper saturated with SDH_2O and allowed to move on this substrate for 10 min to dislodge surface contaminants. At this point, each larva was placed in a tight-lid Petri dish (47-mm diam.) lined with a piece of saturated filter paper (with 400 µl SDH_2O) next to one seven-day-old geranium seedling. One larva was used in each bioassay dish because preliminary experiments revealed that feeding damage inflicted by more than one larva caused significant plant mortality. Two control treatments included individual seedlings with no fungus gnat larvae added and seedlings with one larva treated as described above but not fed on *Pythium*. Seedlings were maintained under standard conditions and watered daily with 200 µl SDH_2O. Mortality was recorded after 7 days. *P. aphanidermatum* was re-isolated from infected seedlings at the end of the experiment.

Two assays (20 replicates/treatment) of the aforementioned experiment were conducted with half-strength corn meal agar culture plates of *P. ultimum* strains P4 and PSN-1. Seedlings

were maintained at 25 ± 1ºC and 14h:10h Light:Dark (L:D) and watered daily. Mortality was recorded after 10 days. *P. ultimum* was re-isolated from infected seedlings at the end of the experiment.

Transmission study with seedlings grown in plug mix. Three laboratory bioassays (13-23 replicates/treatment) were conducted to determine whether *P. aphanidermatum* strain K-13 consumed by fungus gnat larvae could be transmitted to cause infection in geranium seedlings rooted in plug mix (see plant propagation section). Fourth-instar larvae were first fed on *P. aphanidermatum* culture plates as described above (2 larvae/dish in this experiment) and then placed on the surface of tap water-saturated plug mix adjacent to a geranium seedling (2 larvae/cell). Plug cells were watered every other day with 1 ml tap water and maintained under standard incubation conditions. Control treatments were the same as those in the petri dish experiment. Individual plug cells were nested randomly and isolated from one another within an intact, well-drained plug tray. Mortality was recorded after 7 days. *P. aphanidermatum* was re-isolated from infected seedlings at the end of the experiment.

Concentration of *Pythium* spp. oospores and/or hyphal swellings in culture plates. The concentration of oospores and/or hyphal swellings was estimated in twelve culture plates for each *Pythium* species and strain (*P. aphanidermatum* strains Pa58 and K-13 (7-day-old plates) and *P. ultimum* strains P4 and PSN-1 (10-day-old plates)) used in this study. Aerial mycelium was removed from each plate using a sterile scalpel, and the number of total oospores and/or hyphal swellings in focus (within a 0.126 mm^2 area) at the agar surface was counted using 200×

total magnification. Counts were only taken at the agar surface, as preliminary observations revealed that very few spores were formed below this level.

***Pythium* spp. oospore and hyphal swelling germination counts.** Three laboratory bioassays (8-10 replicates/treatment) were conducted to examine the germination rate of *P. aphanidermatum* oospores before and after passage through larval fungus gnats. Germination of oospores before larval passage was determined by using a sterile scalpel to remove 3 mm x 3 mm x 1 mm pieces of agar from *P. aphanidermatum* cultures of both strains K-13 and Pa58. Individual pieces of agar from each plate were placed into separate sterile 1.5-ml microcentrifuge tubes with 150 µl SDH_2O and ground for 30 sec with a sterile 1.5-ml Kontes pellet pestle (Kimble Chase, Vineland, NJ) to dislodge oospores from the agar matrix. The microcentrifuge tubes were then vortexed continuously for 30 sec at fast speed (Scientific Products Deluxe Mixer S8220, McGaw Park, IL) to further disperse the agar and homogenize the suspension. A 50-µl sample of the suspension from each tube was pipetted onto individual water agar (WARP) or nutrient-rich potato dextrose agar (PDA) plates (composed of 6 g PD powder (MP Biomedicals, Solon, OH) and 5 g Bacto^{TM} Agar supplemented with WARP antibiotics in 250 ml SDH_2O) and spread with a sterile glass rod. The proportion of germinating oospores in a 0.875 mm^2 area in the center of each plate was enumerated at 400× magnification after 24 h incubation in the dark at 27 ± 1ºC.

Oospore germination following larval digestion was examined by placing individual fourth-instar larvae on water agar plates for 6 h to clear their guts and then transferring them to V8 agar plates containing a *P. aphanidermatum* culture of either strain K-13 or Pa58 as described for previous experiments. After 15 h, larvae were removed from the culture plates and

transferred onto SDH_2O-saturated sterile filter paper to dislodge surface contaminants. Larvae were then placed individually in 60-mm-diam. petri dishes containing WARP agar or PDA to defecate. After 2 h, larvae were removed from the plates, and the plates were incubated in the dark at 27 ± 1ºC for 24 h. The proportion of germinating oospores in fecal deposits was determined in each plate at 400× magnification.

Additional laboratory bioassays (10 replicates/treatment) of the above-described experiment were conducted to examine the germination rates of *P. ultimum* strain P4 and PSN-1 oospores and/or hyphal swellings on WARP or PDA agar at 25 ± 1ºC before and after passage through larval fungus gnats. Five 5-µl samples of the suspension from each *P. ultimum* control sample were pipetted onto individual WARP or PDA plates (without spreading the sample) for counting. Hyphal swelling germination counts were conducted after 3 h of plate incubation; oospore counts were taken after 24 h.

Statistical analyses. Statistical tests were conducted using the JMP software, version 7.0 (26). Analysis of variance (ANOVA) followed by the Student's t tests for means comparisons were used to examine differences in the proportions of infected seedlings in petri dish experiments and to compare oospore and/or hyphal swelling germination rates and densities of spores in plates. All proportions were arcsine transformed. The log (X + 1) transformation was applied to spore concentrations prior to ANOVA to normalize the distributions and equalize the treatment variances.

RESULTS

Transmission study with seedlings in plastic dishes and plug mix. In petri dishes, plant death due to larval fungus gnat transmission of *Pythium* was greatest (65%) when the insects were fed on *P. aphanidermatum* strain K-13 prior to their placement near seedlings. In plug cells, however, few seedlings died (6%) when exposed to larvae that had fed on strain K-13 (Table 4.1). Larvae were less efficient at transmitting the other three strains; in the petri dish assays, *P. aphanidermatum* strain Pa58 and *P. ultimum* strain P4 were not transmitted (no plant mortality) and *P. ultimum* strain PSN-1 killed only 8% of the seedlings. No seedlings died in the seedling-only controls, and few seedlings died due to fungus gnat feeding alone (<2% only in petri dish assays) (Table 4.1; Figure 4.1).

Concentration of *Pythium* spp. oospores and hyphal swellings in culture plates. Larvae were exposed to significantly more oospores per square millimeter when feeding on *P. aphanidermatum* strain K-13 than strain Pa58 culture plates ($F_{[1,22]} = 15.4$, $P = 0.0007$) (Table 4.2). *Pythium aphanidermatum* strain K-13 oospores were similar in size (21.6 $\pm$ 0.4 µm in diameter; n=10) to those described by Matthews (23) and Van der Plaats-Niterink (30) and possessed distinct cell walls (1.7 $\pm$ 0.05 µm thick; n=10). Only a few mature oospores were ever observed in *P. aphanidermatum* strain Pa58 plates, although developing oospores were frequently observed within oogonia (Figure 4.1, Figure 4.2).

There was no significant difference in the mean number of propagules (oospores and/or hyphal swellings) produced by *P. ultimum* strains P4 and PSN-1 ($F_{[1,22]} = 0.20$, $P = 0.66$). *Pythium ultimum* strain PSN-1 produced both oospores and hyphal swellings (Table 4.2). The oospores measured 20.5 $\pm$ 0.9 µm in diameter (n=10) with a wall thickness of 1.5 $\pm$ 0.1 µm (n=10), while the hyphal swellings were 23.3 $\pm$ 1 µm (n=10) in diameter with a wall thickness of

Table 4.1. Larval fungus-gnat-mediated transmission of *Pythium* spp. in laboratory assays with geranium seedlings.

Treatment	No. of Assays	Total No. of Plants	Mean Plant Mortality (%) (range over assays) [a]
Petri Dishes			
Pythium aphanidermatum			
Control	3	56	0
Fungus Gnats	3	56	1.67 ± 1.67 a (0–5)
Fungus Gnats + *P. aphanidermatum* Pa58	3	56	0
Fungus Gnats + *P. aphanidermatum* K-13	3	56	65.42 ± 7.37 b (56.25–80)
Pythium ultimum			
Control	2	40	0
Fungus Gnats	2	40	0
Fungus Gnats + *P. ultimum* P4	2	40	0
Fungus Gnats + *P. ultimum* PSN-1	2	40	7.5 ± 3.54 (5–10)
Plug Cells			
Pythium aphanidermatum			
Control	3	40	0
Fungus Gnats	3	50	0
Fungus Gnats + *P. aphanidermatum* K-13	3	50	5.68 ± 2.84 (0–8.70)

[a] Percentages followed by same letter are not significantly different ($F_{[1,4]} = 62.4$, P = 0.0014; Student's t test; alpha = 0.05).

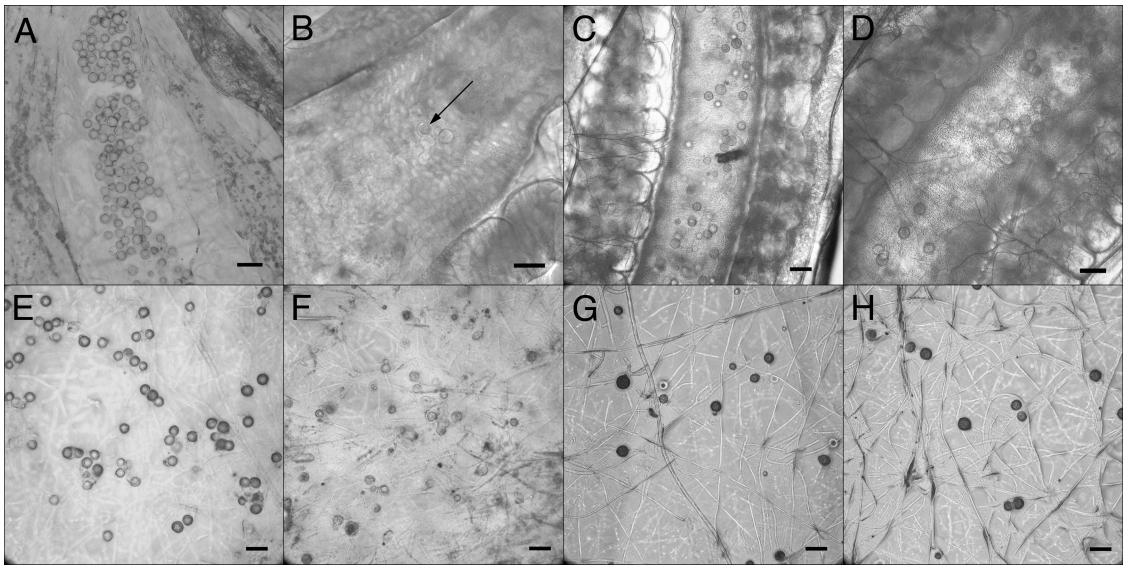

Figure 4.1. **A–D**, Oospores of *P. aphanidermatum* strain K-13 and *P. aphanidermatum* strain Pa58, oospores and hyphal swellings of *P. ultimum* strain PSN-1, and hyphal swellings of *P. ultimum* strain P4, respectively, in the digestive tracts of fungus gnat larvae. Arrow indicates oospore. **E–H**, Surface of *P. aphanidermatum* strain K-13, *P. aphanidermatum* strain Pa58, *P. ultimum* strain PSN-1, and *P. ultimum* strain P4 culture plates, respectively, where fungus gnat larvae were placed to feed. Scale bars = 50 µm.

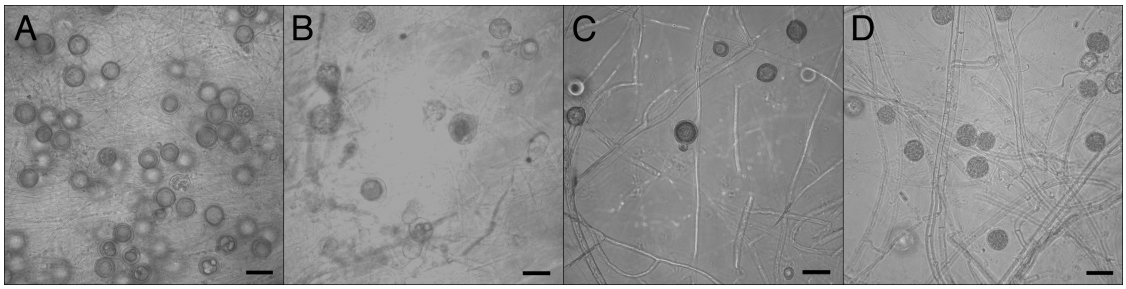

Figure 4.2. **A–D**, Surface of *P. aphanidermatum* strain K-13, *P. aphanidermatum* strain Pa58, *P. ultimum* strain PSN-1, and *P. ultimum* strain P4 culture plates, respectively. Scale bars = 30 µm.

Table 4.2. Mean number of oospores in *Pythium aphanidermatum* and oospores and/or hyphal swellings in *P. ultimum* culture plates.

Treatment	Propagule Type	Total No. of Plates	Mean Number of Propagules/mm^2 (range) [a]
Pythium aphanidermatum			
Strain Pa58	oospores	12	130.3 ± 19.3 a (40–302)
Strain K-13	oospores	12	240.1 ± 22.8 b (135–405)
Pythium ultimum			
Strain P4	hyphal swellings	12	52.9 ± 6.6 a (16–87)
Strain PSN-1	oospores and hyphal swellings	12	47.6 ± 5.7 a (16–79)

[a] Percentages followed by same letter within a species are not significantly different (Student's t test; alpha = 0.05).

1.3 ± 0.1 µm (n=10) (5, 28). *Pythium ultimum* strain P4 produced hyphal swellings only, and these were similar in size (22.8 ± 0.7 µm; n=10) and wall thickness (1.4 ± 0.1 µm; n=10) to those produced by strain PSN-1 (Table 4.2, Figure 4.1, Figure 4.2).

***Pythium* spp. oospore and hyphal swelling germination counts.** Nearly three times more oospores of *P. aphanidermatum* strain K-13 germinated after passage through larval fungus gnats (19%) than without larval gut passage (7%). There was no effect of growth medium (PDA vs. WARP) on germination and no medium x gut passage interaction (Table 4.3). No oospores of *P. aphanidermatum* strain Pa58 germinated with or without passage through larval fungus gnats (Table 4.3). Few oospores of *P. ultimum* strain PSN-1 germinated after larval gut passage (4%), but there was no germination of spores without larval passage.

Significantly fewer hyphal swellings of *P. ultimum* strains PSN-1 and P4 germinated after passage through larval fungus gnats. Gut passage reduced viability of strain PSN-1 from 88 to 37% and viability of strain P4 from 90 to 4% (Table 4.3). As observed with oospores, there were no significant main effects of medium on hyphal swelling germination. With strain P4, there was a weakly significant interaction (P=0.02) between growth medium and gut passage, with a greater reduction in viability observed on WARP agar; however, the main effect of greatly reduced viability after gut passage was evident regardless of the medium used.

DISCUSSION

Relatively few studies have examined the importance of larval fungus gnats in the transmission of plant pathogens (7, 10, 16, 17), and ours is the first to consider the importance of plant growing medium. We observed high levels of transmission (65% seedling mortality) in our

Table 4.3. Mean percentage of germinating *Pythium aphanidermatum* oospores and *P. ultimum* oospores/hyphal swellings with or without passage through larval fungus gnat guts.

Treatment	Total No. Replicates	Total No. Counted Propagules	Percent Germination[a]	ANOVA[b]
Pythium aphanidermatum				
Strain Pa58 (Oospores)				
Control on PDA	8	21	0	
Control on WARP	28	44	0	
Mean germination without gut passage			0	
Fungus gnat larvae on PDA	8	6	0	
Fungus gnat larvae on WARP	28	20	0	
Mean germination after gut passage			0	
Strain K-13 (Oospores)				
Control on PDA	8	29	6.9 ± 4.2	M: $F_{[1,58]} = 0.0007$; $P = 0.98$
Control on WARP	28	196	7.14 ± 1.85	GP: $F_{[1,58]} = 15.00$; $P =$
Mean germination without gut passage			7.11 ± 1.66 a	0.0003
Fungus gnat larvae on PDA	8	123	19.51 ± 3.28	M x GP: $F_{[1,58]} = 0.16$; $P =$
Fungus gnat larvae on WARP	28	459	18.3 ± 2.13	0.69
Mean germination after gut passage			18.56 ± 1.79 b	
Pythium ultimum				
Strain PSN-1 (Oospores)				
Control on PDA	10	32	0	
Control on WARP	20	42	0	
Mean germination without gut passage			0	
Fungus gnat larvae on PDA	10	34	5.88 ± 3.67	
Fungus gnat on WARP	20	57	3.51 ± 1.49	
Mean germination after gut passage			4.4 ± 1.59	
Strain PSN-1 (Hyphal Swellings)				
Control on PDA	10	41	85.37 ± 4.85	M: $F_{[1,30]} = 0.44$; $P = 0.51$
Control on WARP	10	54	90.74 ± 5.43	GP: $F_{[1,30]} = 44.08$; $P =$
Mean germination without gut passage			88.42 ± 3.62 a	<0.0001
Fungus gnat larvae on PDA	10	52	48.08 ± 8.31	M x GP: $F_{[1,30]} = 2.85$; $P =$
Fungus gnat larvae on WARP	10	70	28.57 ± 8.57	0.10
Mean germination after gut passage			36.88 ± 6.29 b	
Strain P4 (Hyphal Swellings)				
Control on PDA	10	230	85.65 ± 1.95	M: $F_{[1,30]} = 0.0032$; $P = 0.95$
Control on WARP	10	355	92.39 ± 1.36	GP: $F_{[1,30]} = 499.82$; $P =$
Mean germination without gut passage			89.74 ± 1.35 a	<0.0001
Fungus gnat larvae on PDA	10	52	9.61 ± 4.58	M x GP: $F_{[1,30]} = 6.33$; $P =$
Fungus gnat larvae on WARP	10	174	2.87 ± 0.63	0.02
Mean germination after gut passage			4.42 ± 1.73 b	

[a] Percent germination (± standard error) determined after incubation of oospores for 24 h and incubation of hyphal swellings for 3 h. Means within strains followed by same letter are not significantly different (Student's t test; alpha = 0.05).

[b] Factorial analysis of variance (ANOVA) testing main effects and interaction; M = effect of culture growth medium on oospore germination, GP = effect of gut passage on germination, M x GP = interaction of medium and gut passage on oospore or hyphal swelling germination.

petri dish assays with *P. aphanidermatum* strain K-13 but very low levels (<6% mortality) in the plug-cell assays with a peat-based potting medium. The very different physicochemical properties of the filter paper vs. potting medium substrates may be largely responsible for the these results, but it is also possible that the microbial composition of the non-sterilized plug mix influenced the level of observed *Pythium* infection. *Pythium* suppressive characteristics have been reported for some peat-based potting mixes (15, 29). Regardless of the inhibition mechanism(s), these findings have important implications with respect to the potential for larval fungus gnats to transmit *Pythium* to plants potted in commercial growing media.

Our study indicates also that the capacity to be vectored by larval fungus gnats varies greatly among *Pythium* species and strains and provides evidence that this variability derives, in large part, from differences in the number and nature of the propagules produced by each pathogen. Transmission rate was high with *P. aphanidermatum* strain K-13, which produced the greatest number of oospores per plate, and low with *P. ultimum* strain PSN-1, which produced substantially fewer oospores. Additionally, transmission occurred only with those *Pythium* strains that produced numerous mature (thick-walled) oospores. *Pythium aphanidermatum* strain Pa58, a strain that produced many oogonia and immature oospores but few mature oospores was not transmitted (abortive oospore development is a common phenomenon among *Pythium* isolates (see 30)). Finally, assays revealed low transmission of oospore-producing strain PSN-1 of *P. ultimum*, but no transmission of *P. ultimum* P4, a strain that produced abundant hyphal swellings but no oospores.

Our finding of no transmission of strain P4 is in sharp contrast to the observations of Hyder et al. (16), who reported high rates of infection (67%) among pepper seedlings exposed to larvae fed on *P. ultimum* hyphal swellings. This disparity could be the result of numerous

factors, including differences in virulence of the pathogen strains, susceptibility of the host plants, bioassay methods, and propagule concentrations. It is particularly noteworthy that the number of hyphal swellings in the larval gut illustrated in Hyder et al. (16) is much higher than any number we observed in our assays (see Figure 4.1, 4.2). It is also possible, however, that the hyphal swellings produced by our strains of *P. ultimum* were less robust (less resistant to digestion) or were retained in the fungus gnat digestive tracts for longer times than those in the study by Hyder et al. (16). In our tests, gut passage reduced the viability of hyphal swellings of strains PSN-1 and P4 from 88 to 37% and 90 to 4%, respectively (Table 4.3). Unfortunately, the results reported by Hyder et al. (16) do not include rates of germination for comparison.

With regard to germination potential of *Pythium* propagules, our findings of limited, slow germination of freshly harvested oospores (maximum 20% germination within 24 h) and extensive, rapid germination of hyphal swellings (maximum 92% germination within 3 h) are in accord with previous studies (27, 28). Air-drying or aging of oospores for 1-2 weeks in nonsterile soil extract has been shown to significantly increase rates of germination (to approximately 90%) for both *P. aphanidermatum* and *P. ultimum* (1, 18, 25, 28). The loss of endogenous carbon over longer time periods correlates negatively with the germinability and virulence of oospores (24). Perhaps the *Pythium* spp. oospores that did not germinate in our study were not mature or given sufficient time and appropriate drying conditions to break dormancy and achieve maximum germination potential. Other factors to consider in oospore germination include soil temperature, pH, and moisture content, and the values of these parameters vary depending on *Pythium* spp. (1, 24).

We did not examine the physiological factors underlying increased germination of *P. aphanidermatum* strain K-13 oospores following fungus gnat larval gut passage and cannot

suggest a mechanism beyond previously stated hypotheses. This is not the first report of this phenomenon; Stanghellini and Russell (27) reported that passage of *P. aphanidermatum* oospores through live water snails increased germination of the spores to 94% and suggested that "enzymes present in the snail intestine increased oospore permeability which allowed the diffusion of nutrients required for subsequent germination" (27). Such marked changes in germination following gut passage could obviously influence vectoring potential, and additional studies are needed to better understand biological factors mediating oospore and hyphal swelling germination and the numbers of these propagules necessary to cause plant infection.

Laboratory studies have now revealed that larval fungus gnats can serve as vectors of certain *Pythium* spp. and strains under ideal conditions (16, 17), but the vectoring potential of these pests in the commercial greenhouse setting remains unexplored. As these insects are slow moving cryptic inhabitants of soil, it seems highly unlikely that they play any significant role in vectoring *Pythium* between greenhouse facilities, between benches in a greenhouse, or between separated pots on a greenhouse bench. Transmission would be most likely among multiple plants within a single pot or hydroponic trough or between cells of propagation trays or seedling flats. But, under these circumstances, *Pythium* spp. are fully capable of unassisted movement (via zoospores or hyphal growth). In addition, while rates of transmission are almost certainly dependent upon propagule concentration in the fungus gnat gut, concentrations as high as those shown in Fig 4.1 of this study or Fig. 4A of Hyder et al. (16) would probably be rare under natural conditions (we observed only sparse production of oospores and/or hyphal swellings in diseased roots of geranium seedlings).

The findings of this and previous studies on *Bradysia-Pythium* interactions (citations) as well as various characteristics of fungus gnat biology and behavior lead us to conclude that these

insects are not important vectors of *Pythium* spp. Fungus gnat management should not therefore be considered an essential part of the *Pythium* disease control equation (fungus gnats need not be intensively controlled out of fear of vectored *Pythium* diseases). This is not to suggest, however, a return to old views of fungus gnats as primarily nuisance pests, as the larvae can sever primary roots or tunnel into the stems of small plants with lethal effects, and adults can mechanically transmit many virulent plant-pathogenic conidial fungi (2, 7, 8, 9, 11, 13, 19). Overall, this study emphasizes the importance of examining the basic biology of fungus gnats and *Pythium* spp. to better understand the ways in which they interact.

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CHAPTER 5: Fungus gnat feeding and mechanical wounding inhibit *Pythium aphanidermatum* infection of geranium seedlings

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ABSTRACT

Braun, S.E., Sanderson, J.P., Nelson, E.B., Daughtrey, M.L., and Wraight, S.P. 2009. Fungus gnat feeding and mechanical wounding inhibit *Pythium aphanidermatum* infection of geranium seedlings. Phytopathology 99: 1421-1428.

A series of laboratory tests were conducted to investigate potential effects of fungus gnat (*Bradysia impatiens*) feeding damage on susceptibility of geranium seedlings (*Pelargonium* x *hortorum*) to infection by the root rot pathogen *Pythium aphanidermatum*. Effects were compared with those from similar tests in which the seedlings were mechanically wounded by severing the root tip with a scalpel. Assays of geranium seedlings in petri dishes revealed a pronounced negative fungus gnat – *Pythium* interaction, with exposure to fungus gnat larvae 24 h prior to inoculation with *P. aphanidermatum* zoospores resulting in up to 47% fewer seedling deaths than would have been expected if the two agents had acted independently. Similar results were observed when seedlings were subjected to mechanical wounding 24 h prior to zoospore inoculation. In contrast, no interaction occurred when seedlings were mechanically wounded immediately prior to inoculation. The degree of plant damage inflicted by the feeding activities

of the larval fungus gnats had no significant effect on the combined damage from fungus gnats and *Pythium* in Petri dishes. Ancillary studies showed that *Pythium* development on V8 agar was not inhibited by presence of fungus gnat-associated microorganisms, nor were seedlings inoculated with these microbes less susceptible to *Pythium* infection. The precise mechanism or mechanisms underlying the observed interactions were not elucidated; however, the results strongly suggest that both fungus gnat feeding and mechanical wounding activated systemic defenses that made the seedlings more resistant to *Pythium* infection.

INTRODUCTION

Plants are constantly threatened by a number of biotic stresses. Plant response to herbivore or pathogen attack is variable in space and time and depends on the ability of a plant to respond with induced resistance or to tolerate attacking organisms. Abiotic and ontogenetic factors influence plant-antagonist interactions as well. Plants are often attacked by more than one organism at a time, forcing them to mount an integrated system of defense against diverse enemies. The order in which different antagonists associate with a plant affects the type and degree of response (5, 24, 35).

Plant pathogens vectored by insects can have devastating effects on plants. This association of insects with plant pathogens has been documented for more than a century (39). In commercial greenhouses, insects previously considered nuisances have been implicated in the spread of soilborne pathogens. One key example of such insect pests is a dark-winged fungus gnat of the genus *Bradysia* (Diptera: Sciaridae) (19). Adult fungus gnats transmit a variety of plant pathogens that produce aerial dispersal stages, including spore-producing fungi such as *Fusarium avenaceum* (Fr.) Sacc., *F. foetens* Schroers O'Donnell, Baayen & Hooftman, *F.*

oxysporum f. sp. *radicis-lycopersici* Jarvis & Shoemaker, *Thielaviopsis basicola* (Berk. & Broome) Ferraris, and *Verticillium albo-atrum* Reinke & Berthold (8, 9, 12, 18, 23). Wounding by fungus gnats may also influence pathogen infection (19). Leath and Newton (25) found that alfalfa and red clover plants injured by fungus gnat (*Bradysia* sp.) larvae prior to inoculation with *Fusarium* spp. died at a much higher rate than uninjured plants. A possible correlation between *Bradysia* fungus gnat infestations and root rot diseases caused by *Pythium* has also been observed. Plants infected with *Pythium* are often also infested with fungus gnat larvae. Both of these organisms are important pests of greenhouse floriculture, and it is generally thought that fungus gnats vector *Pythium*. However, interactions among fungus gnats, *Pythium*, and floral crops have not been thoroughly investigated experimentally (11, 21). The primary objective of this study was to determine if wounding damage inflicted by fungus gnat larvae affects susceptibility of geranium seedlings (*Pelargonium* x *hortorum* L.H. Bailey) to infection by *Pythium aphanidermatum* (Edson) Fitzp. Additional objectives included determining whether *P. aphanidermatum* infection is impacted by the microfloral community associated with fungus gnats or by mechanical wounding. Preliminary studies were also conducted to estimate the median lethal concentration (LC_{50}) of *P. aphanidermatum* zoospores against geranium seedlings in the bioassay systems to be described. This information was needed to determine an appropriate target zoospore dose for tests involving single-dose inoculations, which were part of the main objectives.

MATERIALS AND METHODS

Insect Rearing. A laboratory colony of *Bradysia impatiens* (Johannsen) was established with adults collected from a greenhouse on the Cornell University campus. Adults and larvae were routinely reared in plastic containers (9.8 cm in diameter by 6.2 cm in depth; Pioneer Plastics, Dixon, KY) with lids bearing holes (5.5 cm in diameter) covered with nylon mesh (95 μm) for ventilation. Fifty fungus gnat adults (predominately females) were placed in each container for egg production and provided with ≈40 g of growing medium (Premier Pro-Mix BX, Quakertown, PA) saturated with tap water and ≈10 g (dry weight) of ground pinto beans (Goya Foods, Inc., Secaucus, NJ). Colony containers were maintained at 27 ± 1ºC with 14 h of light and 10 h of darkness. New containers were established daily to provide a constant supply of all life stages. To obtain an even-aged cohort of fourth-instar fungus gnat larvae for use in bioassays, ≈200 adult fungus gnats were collected from colony containers and released into a 45-by-45-by-45.5-cm cage in the laboratory at ambient temperature (≈24ºC) and allowed to oviposit on 90-mm-diameter petri dishes (Becton Dickinson Labware, Franklin Lakes, NJ) that contained saturated cotton covered with a piece of black filter paper spread with a thin layer (≈1.6 g) of ground pinto beans (*Phaseolus vulgaris* L.). After 24 h, petri dishes were removed from cages and the fungus gnat cultures maintained until the larvae completed development to the fourth instar. Additional water and pinto beans were added to the dishes as needed to provide adequate food and water. Fourth-instar larvae were readily identified on the surface of the filter paper and transferred to bioassay containers using a fine brush.

Plant Propagation. Assays were conducted in petri dishes for simplicity and careful observation; other assays were conducted with vermiculite growing medium in plug cells to simulate commercial growing conditions. Geranium seeds (*Pelargonium* x *hortorum* 'Orbit

White', Goldsmith Seeds) for petri dish bioassays were surface sterilized in a 0.5% sodium hypochlorite solution for 3 min, rinsed in sterile distilled water (SDH_2O), and germinated individually on top of one disk of filter paper (42.5 mm in diameter) saturated with 400 µl of SDH_2O in tight (friction)-lid petri dishes (47 mm in diameter). The seeds were incubated in darkness for 3 days at room temperature. Seedlings were maintained at 27 ± 1ºC with 14 h of light and 10 h of darkness and watered daily (with 200 µl of SDH_2O) until they were used in bioassays. The seedlings were oriented horizontally in the petri dishes (lying on the filter paper).

Seeds used in vermiculite/plug tray bioassays were also surface sterilized (as described above) and germinated on top of two disks of filter paper (90 mm in diameter) saturated with 2.2 ml SDH_2O in petri dishes (90 mm in diameter) sealed with Parafilm. The seeds were incubated in darkness at room temperature until germination (3 to 4 days) and then planted in water-saturated, coarse vermiculite within individual plug cells (≈26-ml volume) that were cut from 128-well plug trays (Dillen Products/Myers Industries Inc., Middlefield, OH). Plug cells were placed in 55-ml plastic vials and SDH_2O was added at rates described below for each experiment. Vermiculite was selected as the potting medium because *Pythium* infection was not consistent in a variety of peat-based plug mixes commonly used by growers.

At the initiation of bioassays, seedlings were, in most cases, 7 to 8 days old (days post seed hydration), ≈4 cm in length, and consisted of short hypocotyls terminating in two cotyledons and an unbranched root with dense root hairs.

Pathogen Maintenance. *P. aphanidermatum* (strain Pa58) cultures were grown in 60-mm-diameter petri dishes on V8 juice agar (composed of 100 ml of V8 juice, 400 ml of H_20, 1.5 g of $CaCO_3$, and 10 g of Bacto Agar) and maintained in the dark at 27 ± 1ºC. One-week-old cultures

of *P. aphanidermatum* were used in all experiments. Every 2 weeks, the pathogen was re-isolated from 11-day-old geranium seedlings that had been inoculated 3 days earlier by placing a 6-mm-diameter agar disk (from 1-week-old culture) of *P. aphanidermatum* on the root tip. Each infected seedling was placed in a 90-mm-diameter petri dish and covered with 2% molten water agar supplemented with the antibiotics rifampicin, penicillin G, and kanamycin (at 1.0, 1.0, and 2.5 ml/liter of SDH_2O, respectively) (WARP). After the agar solidified, plates were incubated as described above. *P. aphanidermatum* hyphae that grew rapidly through the agar were excised from the surface of the medium and transferred to a fresh WARP plate. In the following descriptions of methods, the term "replicate assays" refers to assays conducted on different days, using freshly prepared *P. aphanidermatum* inocula.

Inoculum Preparation. Zoospore suspensions were produced by placing one 20-mm-diameter agar disk from a 1-week-old *P. aphanidermatum* culture into a 60-mm petri dish containing 10 ml of SDH_2O. Zoospore culture plates were started at 1700 h on the day prior to the start of an assay. The dish was sealed with parafilm and incubated as described above. The sterile water was replaced with 10 ml of fresh SDH_2O the following morning and zoospores were collected 6 h later for use in the assay.

Approximate desired concentrations of zoospores for bioassays were obtained by preparing serial dilutions in SDH_2O. Concentrations of zoospores in stock suspensions and initial dilutions in a series were estimated by standard hemacytometer counting. Four independent samples from each dilution were counted. Numbers of zoospores in dilute suspensions were determined by spreading a 200-µl sample onto V8 agar in a 90-mm-diameter

petri dish (three replicate plates per dilution). CFU were counted on each plate after 24 h incubation as described above.

***P. aphanidermatum* LC_{50} estimations.** Four replicate multi-dose petri dish bioassays were conducted to determine the LC_{50} of *P. aphanidermatum* zoospores. Four zoospore dose levels and a sterile water control were used in each assay. For the first three assays, the zoospore stock suspension was serially diluted 10x, 10x, 3x, and 3x. In the fourth assay, the stock suspension was serially diluted 10x, 5x, 3x, and 3x. Individual seedlings in tight-lid petri dishes (47 mm in diameter) were inoculated by pipetting 400 µl of one of the four zoospore dilutions onto the filter paper adjacent to the root tip of the plant. Controls were inoculated with 400 µl of SDH_2O. In all, 100 seedlings were inoculated per assay (20 per zoospore dose and control). Mortality of seedlings was recorded 7 days postinoculation.

Five replicate multidose bioassays were conducted to determine the LC_{50} of *P. aphanidermatum* zoospores against geranium seedlings rooted in vermiculite. For all assays, three 5x dilutions with SDH_2O were made to the zoospore stock suspension. Individual 8-day-old geranium seedlings in vermiculite-filled plug cells were inoculated by pipetting 2 ml of either one of the four zoospore suspensions or sterile water near the crown of the plant. In all, 65 to 90 seedlings were used per assay (13 to 18 per zoospore dose and control). The seedlings were maintained at 27 ± 1ºC with 14 h of light and 10 h of darkness and watered daily with 1 ml of sterile water. Mortality of seedlings was recorded 7 days postinoculation.

Effect of fungus gnat wounding on *Pythium* infection. In total, 24 assays were conducted to determine the effects of plant wounding on subsequent *P. aphanidermatum* infection of

geranium seedlings growing on water-saturated filter paper in petri dishes. Twenty-one of these assays included treatments designed to determine whether the time of seedling exposure to fungus gnat larvae influenced infection. Each assay was designed as a two-by-two factorial with all combinations of fungus gnat larval presence or absence and *P. aphanidermatum* inoculum presence or absence as the four treatments (see statistical analysis section). Geranium seed were germinated as described above. When the seedlings were 7 days old, fourth-instar fungus gnat larvae were placed in half of the dishes (three larvae/dish) and allowed to feed on the seedlings. The plants were exposed to the larvae for 24 h at 27 ± 1ºC with 14 h of light and 10 h of darkness, after which time the larvae were removed. Preliminary experiments demonstrated that fungus gnats reliably caused plant damage that was visible by 24 h. Fungus gnat damage was assessed on each plant according to the following scale: 1 = no visible damage or damaged root hairs or minor abrasions; 2 = clearly evident feeding damage scarring the root, hypocotyl, or cotyledon surface; and 3 = severed root, tunneling in stem or root column, hole in cotyledon. Dishes to be treated with *P. aphanidermatum* were then inoculated by pipetting 400 µl of a *P. aphanidermatum* zoospore suspension (150x dilution of stock suspension) onto the filter paper near the root tip of the plant. Doses (CFU) applied in each of the replicate assays were quantified using the above-described CFU plating method. Each treatment included 20 to 25 seedlings. Mortality of seedlings was recorded 7 days postinoculation.

Four laboratory bioassays were conducted to determine the effects of exposure to fungus gnat larvae on subsequent *P. aphanidermatum* infection of geranium seedlings rooted in vermiculite-filled plug cells contained in plastic vials (see plant propagation section). Each assay was designed as a two-by-two factorial with all combinations of fungus gnat larval presence or absence and *P. aphanidermatum* inoculum presence or absence as the four treatments. Fourth-

instar fungus gnat larvae were then placed in half of the cells (three larvae/cell) and allowed to feed on the 7-day-old seedlings. At 24 h after introduction of the larvae, the dishes were inoculated with *P. aphanidermatum* by pipetting 2 ml of a stock zoospore suspension onto the vermiculite adjacent to the crown of the plant. Doses applied in each of the four replicate assays were quantified by hemacytometer. In all, 68 seedlings were used per assay (17 per treatment). The seedlings were maintained at 27 ± 1ºC with 14 h of light and 10 h of darkness. At initiation of the assays, SDH_20 was added to each vial to a level 1 cm above the bottom of the plug cell, and water was subsequently added once daily to restore this level. Mortality of seedlings was recorded 7 days postinoculation.

Effect of mechanical wounding on *Pythium* infection. Of the above-mentioned 24 petri dish assays, 14 included treatments to determine the effects of mechanical wounding of plants on subsequent *P. aphanidermatum* infection of geranium seedlings. Each assay was designed as a two-by-two factorial with all combinations of mechanical wounding presence or absence and *P. aphanidermatum* presence or absence as the four treatments. Mechanical wounding was achieved by cutting a 1-cm-long section from the distal portion of the root of each seedling using a sterilized scalpel. In each of the 14 assays, *P. aphanidermatum* zoospores were applied to 8-day-old seedlings immediately after wounding. In 9 of the 14 assays, zoospores were applied to seedlings that had been wounded 24 h previously (when 7 days old). Twenty seedlings were used per treatment. Mortality of seedlings was assessed 7 days postinoculation.

Effect of fungus gnat-associated microbes on *Pythium* infection. Four bioassays were conducted to determine the possible effects of the fungus gnat larval microbial community on *P.*

aphanidermatum infection of geranium seedlings. Fungus gnat rinsate was generated by placing three fourth-instar fungus gnat larvae in a sterile 2-ml centrifuge tube with 10 µl of sterile water. The tube was then vortexed for 30 s to remove microorganisms from the fungus gnats' bodies. Eighty 7-day-old seedlings per assay were treated by applying 10 µl of fungus gnat rinsate via pipette either to the crown or to the root tip of a seedling (20 seedlings/treatment). In corresponding control treatments, SDH_2O was applied instead of rinsate. Three replicate 200-µl samples of each rinsate (diluted 2,000x) were also pipetted onto V8 agar plates for CFU counts. One day after application of the rinsate treatments, the seedlings were inoculated with *P. aphanidermatum* zoospores and monitored for mortality as described above.

An additional assay was conducted in vitro to assess possible antagonistic effects of the fungus gnat-associated microbes on *P. aphanidermatum* infection. One 6-mm-diameter agar disk from a 1-week-old culture of *P. aphanidermatum* was placed on the surface of V8 medium in the center of each dish. In one treatment, three 10-µl droplets of fungus gnat rinsate were applied individually along the circumference of the dish equidistant from the *P. aphanidermatum* plug. In the second treatment, three 10-µl droplets of rinsate were applied individually to 3-day-old geranium plants situated along the circumference of a 90-mm plate equidistant from the *P. aphanidermatum* plug. Plates were maintained at 27 ± 1ºC with 14 h of light and 10 h of darkness, and growth of the *Pythium* sp. was examined 3 days post-inoculation. Twelve replicates of each treatment were used. In three replicates, 200-µl samples of the fungus gnat rinsate (diluted 8,000x) were also pipetted onto individual V8 agar plates (90-mm-diameter petri dish) for CFU counts.

Statistical Analyses. LC_{50}s of *P. aphanidermatum* zoospores were estimated by probit analysis using the personal computer program PoloPlus (26). All other statistical tests were conducted using the JMP software version 7.0 (36) using analysis of variance (ANOVA) and significance levels of $\alpha = 0.05$. All percent mortality data were arcsine transformed prior to parametric ANOVA (37) and aligned-rank transformed for the alternative nonparametric test (27, 33). It was not possible to exactly replicate zoospore dose across assays. Therefore, in order to assess the effect of *P. aphanidermatum* dose on infection of geranium seedlings, it was necessary to combine doses into categories. Three categories were defined: < 100 zoospores/ml, 100 to 299 zoospores/ml, or ≥ 300 zoospores/ml. Assay was included as a factor (experimental block) in all analyses but interactions with assay were not examined due to restriction error (37). In addition, because assay was confounded with dose, it was not possible to test dose – treatment interactions.

Of the 24 petri dish bioassays, 14 comprised subexperiments investigating effects of exposure to both fungus gnats and mechanical damage on mortality of *Pythium*-inoculated geranium seedlings. To simplify the interaction analysis and presentation of results, we conducted separate two-by-two factorial analyses of these factors even though independent sets of controls and *Pythium*-only treatments were not conducted for each subexperiment. Though this approach enables a straightforward presentation of results, caution is called for with any sacrifice of independence. We do not consider this a significant problem in this case, because (i) no control mortality was observed in any of the bioassays, indicating no variance associated with the estimate of 0% control mortality, and (ii) trends in the data were consistent across a large number of independently replicated assays, translating in many cases to high levels of significance of the tested interactions (especially the fungus gnat – *Pythium* interactions) (*P* values ≤ 0.001). The lack of control mortality does, however, create a problem with respect to

unequal variance across treatments. As a consequence, we corroborated the ANOVA results using the nonparametric aligned rank test recommended for factorial designs with interaction (27, 33). Results in all cases were similar to the standard ANOVA (often producing increased levels of significance), and we opted to present just the ANOVA results. In addition, we employed one-way ANOVAs to test for independence of the *Pythium* and fungus gnat (or *Pythium* and mechanical damage) mortality factors. Mortality predicted by independent action was calculated using the formula for combination of independent probabilities (34), and this expected mortality was compared directly via ANOVA to the observed mortality caused by the agents acting in combination. This comparison excludes the problematic (zero-variance) control treatments from the analysis and, because control mortality was zero, corrections for control mortality were also unnecessary.

The same problem with independence exists with the tests examining effects of variable fungus gnat damage (separate control and *Pythium*-only treatments were not conducted for each damage level). In this case there were fewer replicates and smaller sample sizes, and we opted not to conduct the factorial analysis. Instead, the fungus gnat + *Pythium* treatments were analyzed by one-way ANOVA (percent mortality data arcsine transformed and weighted by sample size) to determine whether there was a significant effect of fungus gnat damage level on mortality of *Pythium*-inoculated seedlings.

RESULTS

Establishment of *P. aphanidermatum* LC_{50} values. The mean $LC_{50} \pm$ standard error (SE) determined from four replicate bioassays in petri dishes equaled 49.7 ± 15.2 zoospores/ml, with a

probit regression slope ± SE equal to 1.303 ± 0.031. The concentrations of viable zoospores per ml used in the four assays was 0 to 897 and produced a maximum of 100% mortality (Table 5.1). All seedlings remained healthy in the control treatments for the duration of the experiment. Signs of disease in *Pythium*-infected plants included watersoaking that made the tissue appear darker green in color and hyphal growth on the surface of the seedling roots and hypocotyl within 7 days after inoculation.

The mean LC_{50} ± SE determined from five replicate bioassays with seedlings rooted in vermiculite equaled $12.5 \times 10^4 \pm 3.4 \times 10^4$ zoospores/ml; mean probit regression slope ± SE was 1.04 ± 0.381. The concentrations of viable zoospores per milliliter used in the five assays was 70 to 193,000 and produced a maximum of 80% mortality (Table 5.1). Control treatments (SDH_2O) had minimal effect in most cases, with all seedlings in three assays remaining apparently healthy until the assays were terminated. Significant levels of control mortality were recorded in two assays (11 and 22%); however, none of the affected seedlings showed signs of *Pythium* infection, and no *Pythium* sp. was re-isolated from the dead seedlings.

Interaction Studies. Results from the 28 assays investigating effects of plant wounding on *Pythium* infection are presented in Table 5.2. Assays 1 to 24 were conducted with geranium seedlings on wet filter paper in petri dishes, while assays 25 to 28 were conducted with seedlings rooted in vermiculite in plug cells. No seedling mortality was observed in any of the control treatments across assays and, therefore, all mortality in the fungus gnat-alone and *Pythium*-alone treatments was attributable to treatment effects (fungus gnat damage or *Pythium* infection). Interactions were apparent between the main effects of exposure to fungus gnat larvae and

TABLE 5.1. Median lethal concentration (LC_{50}) estimates from replicated four-dose bioassays of *Pythium aphanidermatum* against geranium seedlings in petri dishes and plug trays.

Assay[a]	Dose range (zoospores/ml)[b]	Mortality range (%)[c]	LC_{50} (zoospores/ml)	Probit regression slope	Heterogeneity χ^{2d}
Filter paper					
1	7 – 897	25 – 100	29.8	1.397	3.0
2	0 – 440	5 – 75	94.2	1.275	1.7
3	2 – 583	5 – 95	44.9	1.265	0.2
4	8 – 587	15 – 95	30.0	1.275	2.6
Means ± SE			49.7 ± 15.2	1.303 ± 0.031	
Vermiculite					
1	70 – 31,950	0 – 31	122,168	0.989	0.9
2	342 – 65,800	8 – 46	236,814	0.422	2.1
3	423 – 179,600	27 – 80	20,454	0.473	3.6
4	445 – 85,950	0 – 44	115,922	0.826	3.2
5	538 – 193,000	44 – 78	130,280	2.507	2.0
Means ± SE			125,128 ± 34,315	1.04 ± 0.381	

[a] Seedlings rooted either on moist filter paper in petri dishes (Filter paper) or in vermiculite in plug trays (Vermiculite); SE = standard error.

[b] Range of doses (expressed as viable zoospores per ml) applied in each bioassay.

[c] Range of geranium seedling mortality recorded across doses.

[d] Heterogeneity χ^2 (with 2 degrees of freedom) indicating goodness-of-fit of the data to the probit-log dose linear regression model (10).

TABLE 5.2. Percent mortality of geranium seedlings in 28 damaged-plant assays.

Assay[a]	Dose[b]	Control	FGD[c]	*Pythium*[d]	FGD + *Pythium*[e]	MD[f]	MD + *Pythium*[g]	24-h MD[h]	24-h MD + *Pythium*[i]
Filter paper									
1	68	0	10	25	15	–	–	–	–
2	100	0	0	40	65	–	–	–	–
3	25	0	24	0	16	–	–	–	–
4	128	0	4	8	8	–	–	–	–
5	129	0	4	40	8	–	–	–	–
6	31	0	8	16	8	–	–	–	–
7	111	0	30	30	40	–	–	–	–
8	427	0	10	85	70	–	–	–	–
9	800	0	0	95	60	–	–	–	–
10	168	0	0	65	10	–	–	–	–
11	404	0	0	60	20	0	80	–	–
12	423	0	0	95	35	0	100	–	–
13	194	0	20	85	45	0	85	–	–
14	158	0	10	50	30	0	65	–	–
15	120	0	25	50	35	0	65	–	–
16	187	0	–	80	–	0	40	0	35
17	440	0	–	75	–	0	85	0	30
18	282	0	–	85	–	0	90	0	60
19	113	0	0	15	5	0	35	0	5
20	73	0	0	10	0	0	10	0	0
21	110	0	0	50	10	0	30	0	20
22	322	0	15	95	15	0	95	0	40
23	392	0	5	100	75	0	90	0	60
24	320	0	0	90	55	0	95	0	60
Vermiculite									
25	46100	0	0	65	47	–	–	–	–
26	21925	0	0	18	12	–	–	–	–
27	65825	0	0	35	18	–	–	–	–
28	94450	0	12	24	12	–	–	–	–
29	49425	0	6	35	29	–	–	–	–

[a] Seedling rooted either on moist filter paper in petri dishes (Filter paper) or in vermiculite in plug trays (Vermiculite).
[b] Zoospores per milliliter used in each bioassay
[c] FGD = fungus gnat damage; each seedling exposed to three fourth-instar fungus gnat larvae for 24 h.
[d] Each seedling inoculated with *Pythium* zoospores.
[e] Each seedling exposed to three fourth-instar fungus gnat larvae for 24 h and then inoculated with *Pythium*; larvae removed immediately prior to *Pythium* application.
[f] MD = mechanical damage; each seedling wounded by cutting 1-cm-long section from distal portion of root.
[g] Each seedling wounded by cutting 1-cm-long section from distal portion of root and immediately inoculated with *Pythium* zoospores.
[h] Each seedling wounded by cutting 1-cm-long section from distal portion of root 24 h prior to start of assay.
[i] Each seedling wounded by cutting 1-cm-long section from distal portion of root and inoculated with *Pythium* zoospores 24 h later.

inoculation with *Pythium* as well as between mechanical damage and *Pythium* inoculation. Evaluation of the significance of these interactions was examined via factorial ANOVA.

Effects of fungus gnat wounding on *Pythium* infection. Exposure to fungus gnat larvae alone did not cause any significant plant mortality, whereas *Pythium* alone was highly virulent when zoospores were applied at the intermediate and high doses (Table 5.3). There was, however, an overall significant interaction between the fungus gnat and *Pythium* treatments. The exposure of seedlings to fungus gnats 24 h prior to *Pythium* inoculation resulted in up to 47% fewer seedling deaths and seedling mortality up to 42 percentage points lower than would have occurred had the two agents operated independently (Table 5.3).

Similar trends were observed with seedlings rooted in vermiculite (Table 5.4). A negative interaction between the fungus gnat and *Pythium* treatments was apparent, with 37% fewer seedling deaths and mortality 14 percentage points lower than predicted by independent action.

There was little evidence that the degree of plant damage inflicted by the feeding activities of the larval fungus gnats had any significant effect on the above-described fungus gnat–*Pythium* interaction (Table 5.5). At the medium zoospore doses, seedling mortality increased with increasing damage (from 14 to 38%, $P = 0.004$); however, this trend was apparent also in the absence of *Pythium*, and significance was lost (P increased to 0.18) when mortality attributable to fungus gnats alone was deducted from mortality in the fungus gnat + *Pythium* treatments using Abbott's formula (1). In the cases of the low and high doses and when all doses were combined, there were no differences in seedling mortality over the three damage levels (Table 5.5).

TABLE 5.3. Effects of inoculation with *Pythium aphanidermatum* and exposure to fungus gnat larvae on mortality (± standard error) of geranium seedlings in petri dish bioassays.

Treatments[a]	Observed Plant Mortality (%)	Expected Plant Mortality (%)[b]	Synergism (+) or antagonism (-)[c]	ANOVA[d]
Controls	0			
PL	12.7 ± 5.0			P: $F_{[1,9]} = 2.73$; $P = 0.13$
FG	10.5 ± 4.0			FG: $F_{[1,9]} = 1.50$; $P = 0.25$
FG + PL	9.7 ± 1.8	22.0	-12.3% (-55.9%)	P x FG: $F_{[1,9]} = 3.06$; $P = 0.11$
				IA: $F_{[1,3]} = 21.7$; $P = 0.019$
Controls	0			
PM	43.3 ± 5.0			P: $F_{[1,27]} = 59.13$; $P < 0.0001$
FG	9.3 ± 3.0			FG: $F_{[1,27]} = 0.11$; $P = 0.74$
FG + PM	25.6 ± 4.3	48.6	-23.0% (-47.3%)	P x FG: $F_{[1,27]} = 11.99$; $P = 0.0018$
				IA: $F_{[1,9]} = 10.66$; $P = 0.0098$
Controls	0			
PH	88.6 ± 3.4			P: $F_{[1,18]} = 220.85$; $P < 0.0001$
FG	3.6 ± 3.1			FG: $F_{[1,18]} = 13.34$; $P = 0.0018$
FG + PH	47.1 ± 6.4	89.0	-41.9% (-47.1%)	P x FG: $F_{[1,18]} = 25.69$; $P < 0.0001$
				IA: $F_{[1,6]} = 31.7$; $P = 0.0013$
Controls	0			
PA	52.6 ± 4.9			P: $F_{[1,60]} = 98.67$; $P < 0.0001$
FG	7.6 ± 3.3			FG: $F_{[1,60]} = 1.50$; $P = 0.23$
FG + PA	29.8 ± 3.0	56.2	-26.4% (-47.0%)	P x FG: $F_{[1,60]} = 17.02$; $P = 0.0001$
				IA: $F_{[1,20]} = 36.12$; $P < 0.0001$

[a] PL = low dose of *Pythium*, $n = 4$ assays, 25 to 73 zoospores/ml; FG = fungus gnats, each seedling exposed to three fourth-instar fungus gnat larvae for 24 h; PM = medium dose of *Pythium*: $n = 10$ assays, 100 to 194 zoospores/ml; PH = high dose of *Pythium*: $n = 7$ assays, 320 to 800 zoospores/ml; PA = all doses; FG + *Pythium* dose = seedlings exposed to fungus gnat larvae for 24 h and then inoculated with *Pythium*; larvae removed immediately prior to *Pythium* application.

[b] Control predicted if the agents exhibit independent action; value calculated using the formula for combination of independent probabilities (34).

[c] Antagonism calculated as the difference between observed and expected percent plant mortality. Numbers in parentheses indicate the proportional differences between the observed and expected percent plant mortality.

[d] Factorial analysis of variance (ANOVA) testing main effects and interaction; one-way ANOVA testing hypothesis of independent action (IA).

TABLE 5.4. Effects of inoculation with *Pythium aphanidermatum* and exposure to fungus gnat larvae on mortality (± standard error) of geranium seedlings rooted in vermiculite.

Treatments	Observed Plant Mortality (%)	Expected Plant Mortality (%)[a]	Synergism (+) or antagonism (-)[b]	ANOVA[c]
Controls	0			
Pythium (P)[d]	35.4 ± 4.8			P: $F_{[1,12]} = 50.8$, $P < 0.0001$
Fungus gnats (FG)	3.6 ± 4.6			FG: $F_{[1,12]} = 0.26$, $P = 0.62$
P + FG	23.6 ± 3.3	37.7	-14.1% (-37.4%)	P x FG: $F_{[1,12]} = 3.40$, $P = 0.09$ IA: $F_{[1,4]} = 27.4$, $P = 0.006$

[a] Control predicted if the agents exhibit independent action; value calculated using the formula for combination of independent probabilities (34).
[b] Antagonism calculated as the difference between observed and expected percent plant mortality. Number in parentheses indicates the proportional difference between the observed and expected percent plant mortality.
[c] Factorial analysis of variance (ANOVA) testing main effects and interaction; one-way ANOVA testing hypothesis of independent action (IA).
[d] Test comprising $n = 5$ assays, 2.2 to 9.4 x 10^4 zoospores/ml.

TABLE 5.5. Effects of fungus gnat (FG) damage level on percent mortality (± standard error) of geranium seedlings exposed to *Pythium aphanidermatum*.

Treatment [a]	FG damage level[b]			Analysis of variance
	1	2	3	
Low dose				
FG	3.8 ± 3.6	3.1 ± 2.7	25.0 ± 5.5	$F_{2,6} = 4.2$, $P = 0.071$
FG + P	8.7 ± 5.5	14.3 ± 5.4	8.7 ± 0.6	$F_{2,6} = 0.5$, $P = 0.62$
Adjusted FG + P	7.7 ± 4.9	12.5 ± 4.8	0	$F_{2,6} = 5.3$, $P = 0.047$
Medium dose				
FG	4.8 ± 4.1	2.9 ± 4.2	17.5 ± 1.8	$F_{2,18} = 2.3$, $P = 0.13$
FG + P	13.6 ± 4.1	15.7 ± 5.4	37.6 ± 2.8	$F_{2,18} = 7.5$, $P = 0.004$
Adjusted FG + P	11.7 ± 4.3	14.5 ± 5.1	26.8 ± 2.6	$F_{2,18} = 1.9$, $P = 0.18$
High dose				
FG	0	0	10.6 ± 2.3	$F_{2,12} = 11.7$, $P = 0.002$
FG + P	39.6 ± 6.3	44.4 ± 6.6	51.1 ± 5.1	$F_{2,12} = 0.9$, $P = 0.44$
Adjusted FG + P	39.6 ± 6.3	44.4 ± 6.6	46.7 ± 5.1	$F_{2,12} = 0.43$, $P = 0.66$
All doses				
FG	2.9 ± 2.1	2.1 ± 1.3	17.0 ± 3.1	$F_{2,51} = 13.5$, $P < 0.0001$
FG + P	22.5 ± 4.9	23.2 ± 6.0	35.0 ± 5.8	$F_{2,51} = 1.5$, $P = 0.24$
Adjusted FG + P	21.5 ± 4.9	22.3 ± 6.1	26.6 ± 6.1	$F_{2,51} = 0.1$, $P = 0.87$

[a] FG: seedlings exposed to FGs alone; FG + P: seedlings exposed to FGs and then treated with *Pythium* sp.; adjusted FG + P: mortality attributable to FGs alone deducted from mortality in the FG + P treatments using Abbott's formula (1). Low dose = 25 to 73 zoospores/ml, 4 bioassays; Medium dose = 100 to 194 zoospores/ml, 10 bioassays; High dose = 320 to 800 zoospores/ml, 7 bioassays; All doses = 21 bioassays.
[b] FG damage was assessed on each plant according to the scale: 1 = no visible damage or damaged root hairs or minor abrasions, 2 = clearly evident feeding damage scarring the root, hypocotyl, or cotyledon surface, 3 = severed root, tunneling in stem or root column, hole in cotyledon.

Effects of mechanical wounding on *Pythium* infection. Mechanical wounding alone did not have a significant effect on plant mortality, whereas *Pythium* sp. alone had a highly significant effect on plant mortality at all doses (Table 5.6). There was no significant interaction between mechanical wounding and *Pythium* treatments (Table 5.6).

In contrast, mechanical wounding of seedlings 24 h prior to *Pythium* inoculation significantly reduced subsequent *Pythium*-induced mortality at the high dose but not at the intermediate or low doses, and a significant interaction was detected between mechanical wounding and *Pythium* (Table 5.6). The exposure of seedlings to mechanical wounding 24 h prior to *Pythium* inoculation resulted in up to 46% fewer seedling deaths and seedling mortality up to 41 percentage points lower than predicted by independent action (Table 5.6).

Effect of fungus gnat microbes on *Pythium* infection. Treating seedlings with microbes washed from fungus gnats had no significant effect on mortality due to *Pythium* infection ($F_{[3,9]} = 1.1$, $P = 0.40$). Percent mortality of seedlings in the rinsate–crown, sterile water–crown, rinsate–root tip, and sterile water–root tip treatments was 46.2 ± 3.1, 51.2 ± 4.4, 52.5 ± 3.2, and 56.2 ± 2.6, respectively. The concentrations of zoospores used in the four assays was 180 to 412 zoospores/ml. The fungus gnat rinsates contained an average of 4.2×10^5 bacteria/ml.
In the presence of fungus gnat rinsates on V8 agar plants, no mycelial inhibition was observed. Furthermore, all of the plants treated with rinsates showed symptoms of *P. aphanidermatum* infection within 2 days. The fungus gnat rinsates contained an average of 1.45×10^5 bacteria/ml.

TABLE 5.6. Effects of mechanical wounding and *Pythium aphanidermatum* dose on geranium seedling mortality (± standard error).

Treatments	Observed Plant Mortality (%)	Expected Plant Mortality (%)[a]	Synergism (+) or antagonism (-)[b]	ANOVA[c]
Damage at inoculation[d]				
Controls	0			
PL	25 ± 8.8			P: $F_{[1,6]} = 25.91$; $P = 0.002$
MD[e]	0			MD: $F_{[1,6]} = 0.003$; $P = 0.96$
PL + MD[f]	25 ± 5.2	25	0% (0%)	P x MD: $F_{[1,6]} = 0.003$; $P = 0.96$
				IA: $F_{[1,2]} = 0.006$, $P = 0.94$
Controls	0			
PM	70 ± 5.9			P: $F_{[1,12]} = 201.83$; $P < 0.0001$
MD[e]	0			MD: $F_{[1,12]} = 0.005$; $P = 0.95$
PM + MD[f]	69 ± 6.4	70	-1% (-1.4%)	P x MD: $F_{[1,12]} = 0.005$; $P = 0.95$
				IA: $F_{[1,4]} = 0.006$, $P = 0.94$
Controls	0			
PH	85 ± 4.3			P: $F_{[1,15]} = 588.75$; $P < 0.0001$
MD[e]	0			MD: $F_{[1,15]} = 0.51$; $P = 0.49$
PH + MD[f]	90.8 ± 1.2	85	+5.8% (+6.8%)	P x MD: $F_{[1,15]} = 0.51$; $P = 0.49$
				IA: $F_{[1,5]} = 0.48$, $P = 0.52$
Controls	0			
PA	66.8 ± 4.3			P: $F_{[1,39]} = 265.84$; $P < 0.0001$
MD[e]	0			MD: $F_{[1,39]} = 0.06$; $P = 0.80$
PA + MD[f]	68.9 ± 4.3	66.8	+2.1% (+3.1%)	P x MD: $F_{[1,39]} = 0.06$; $P = 0.80$
				IA: $F_{[1,13]} = 0.19$, $P = 0.67$
Damage 24 h prior to inoculation[g]				
Controls	0			
PL	25 ± 7.9			P: $F_{[1,6]} = 14.55$; $P = 0.009$
MD[h]	0			MD: $F_{[1,6]} = 2.55$; $P = 0.16$
PL + MD[i]	8.3 ± 1.4	25	-16.7% (-66.8%)	P x MD: $F_{[1,6]} = 2.55$; $P = 0.16$
				IA: $F_{[1,2]} = 29.74$, $P = 0.03$
Controls	0			
PM	82.5 ± 1.2			P: $F_{[1,3]} = 196.44$; $P = 0.0008$
MD[h]	0			MD: $F_{[1,3]} = 9.98$; $P = 0.05$
PM + MD[i]	47.5 ± 8.7	82.5	-35% (-42.4%)	P x MD: $F_{[1,3]} = 9.98$; $P = 0.05$
				IA: $F_{[1,1]} = 16.55$, $P = 0.15$
Controls	0			
PH	88.7 ± 3.8			P: $F_{[1,9]} = 269.18$; $P < 0.0001$
MD[h]	0			MD: $F_{[1,9]} = 20.62$; $P = 0.001$
PH + MD[i]	47.5 ± 4.5	88.7	-41.2% (-46.4%)	P x MD: $F_{[1,9]} = 20.62$; $P = 0.001$
				IA: $F_{[1,3]} = 49.31$, $P = 0.006$
Controls	0			
PA	66.1 ± 6.7			P: $F_{[1,24]} = 98.48$; $P < 0.0001$
MD[h]	0			MD: $F_{[1,24]} = 7.84$; $P = 0.01$
PA + MD[i]	34.4 ± 3.3	66.1	-31.7% (-48.0%)	P x MD: $F_{[1,24]} = 7.84$; $P = 0.01$
				IA: $F_{[1,8]} = 53.33$, $P < 0.0001$

[a] Control predicted if the agents exhibit independent action; value calculated using the formula for combination of independent probabilities (34).
[b] Synergism and antagonism calculated as the difference between observed and expected percent plant mortality. Numbers in parentheses indicate the proportional differences between the observed and expected percent plant mortality.
[c] Factorial analysis of variance (ANOVA) testing main effects and interaction; one-way ANOVA testing hypothesis of independent action (IA).
[d] Mechanical damage (MD) at time of *Pythium* inoculation. PL = low dose: *n* = 3 assays, 73 to 113 zoospores/ml; PM = medium dose: *n* = 5 assays, 120 to 282 zoospores/ml; PH = high dose: *n* = 6 assays, 320 to 440 zoospores/ml; PA = all doses.
[e] Each seedling wounded by cutting 1-cm-long section from distal portion of root.
[f] Each seedling wounded by cutting 1-cm-long section from distal portion of root and immediately inoculated with *Pythium* zoospores.
[g] MD 24 h prior to *Pythium* inoculation. PL = low dose: *n* = 3 assays, 73 to 113 zoospores/ml; PM = medium dose: *n* = 2 assays, 187 to 282 zoospores/ml; PH = high dose: *n* = 4 assays, 320 to 440 zoospores/ml; PA = all doses.
[h] Each seedling wounded by cutting 1-cm-long section from distal portion of root 24 h prior to start of assay.
[i] Each seedling wounded by cutting 1-cm-long section from distal portion of root and inoculated with *Pythium* zoospores 24 h later.

DISCUSSION

The primary goal of this study was to determine if wounding damage from fungus gnat larvae affects the susceptibility of geranium seedlings to infection by *P. aphanidermatum*. The finding that fungus gnat larval feeding reduces infection by *P. aphanidermatum* presents a new and unexpected picture with respect to the role of fungus gnats in the development of plant disease. For example, some previous studies with *Bradysia* spp. have shown that feeding may promote plant infection by pathogenic fungi (15, 25), whereas other studies (12) have demonstrated no enhanced infection from fungus gnat feeding (22). Our results reveal a relationship between fungus gnat feeding and induced resistance to pathogens that has not previously been demonstrated (19).

Fungus gnat larvae have been shown to induce plant defense responses: the jasmonate pathway (16, 20) plays a critical role in protecting *Arabidopsis thaliana* from attack by these insects (29). It is unclear, though, which traits activated by the jasmonate pathway are responsible for increased resistance and whether cross-talk may exist among multiple plant-defense-signaling pathways (20, 29). Furthermore, it is possible that defense responses induced by insect feeding may influence subsequent biotic attackers (5). For example, tomato plants previously infested with silverleaf whitefly, *Bemisia argentifolii*, are more resistant to the fungal pathogen *Erysiphe cichoracearum* than are control plants (28). Because jasmonic acid mediates pathways that confer partial tolerance to necrotrophic pathogens such as *Pythium* (13, 31), it is possible that fungus gnat feeding inhibited *Pythium* infection via the jasmonate response in the current study.

Increasing fungus gnat feeding damage did not increase the level of *Pythium* infection. If increased damage were directly correlated with the level of induced resistance, the expected

result would be decreasing mortality with increasing damage. This was not the case (Table 5.5). These findings agree with recent studies indicating that plants can initiate rapid defense responses within minutes of an insect contacting a plant, and even the nonfeeding activities (movements) of a single insect may initiate a response (17). Furthermore, the production of some plant defensive compounds such as certain terpenoid volatiles and methyl salicylate respond in a nonlinear manner to damage level (30).

Insect feeding and mechanical wounding can induce the same defensive pathways of a plant but the plant response levels and gene transcript profiles may differ (6, 32, 40). In the present study, however, both exposure to fungus gnat larvae and mechanical damage 24 h prior to *Pythium* inoculation resulted in significant reductions in seedling mortality. Explanation for the lack of a significant relationship between plant wounding and *Pythium* infection when mechanical damage was inflicted immediately prior to inoculation may relate to the fact that zoospores are ephemeral, typically encysting and germinating within an hour of locating a host (14). Despite rapid plant defense reactions after herbivore contact, other studies with *Nicotiana sylvestris* reveal that systemic jasmonic acid responses do not reach a maximum until 180 min after wounding (3). In the present study, when wounding was done immediately prior to zoospore inoculation, there may not have been sufficient time for the geranium seedlings to mount a strong defensive response before becoming infected. Considering that fungus gnat larvae are almost always present in the greenhouse setting, plant defense reactions may occur frequently in response to wounding and insect feeding. Plants must regulate these responses, however, to balance defense and fitness costs.

An additional goal of this study was to investigate potential inhibitory characteristics of the fungus gnat-associated microbial community on *P. aphanidermatum* infection.

Microorganisms may play important roles in interactions between macroorganisms and between macroorganisms and their pathogens. For example, microorganisms regurgitated by herbivores have been implicated in the induction of plant defense responses (4, 7, 38). Also, nonpathogenic rhizobacteria have been shown to induce systemic resistance in plants similar to pathogen-induced systemic acquired resistance (SAR). Rhizobacteria-induced systemic resistance has been shown with fungi, bacteria, and viruses on various plants (4). Although microorganisms living in the soil may positively or negatively impact interactions among *Pythium* spp., fungus gnats, and geranium plants, there was no significant impact of the fungus gnats' external microbial community on subsequent *P. aphanidermatum* infection of geranium seedlings in this study.

Our investigations of fungus gnat–*Pythium*–geranium seedling interactions cannot be considered exhaustive. For example, we did not investigate potential inhibitory effects of microbes extracted from the guts of fungus gnats on *P. aphanidermatum* susceptibility. There is also the possibility of fungus gnat larvae directly interacting with plant pathogens and impacting their ability to infect plants. For example, salivary secretions from the fungus gnat, *Bradysia coprophila*, have been shown to inhibit germination of sclerotia from the plant pathogen *Sclerotinia sclerotiorum* (2). Also, larvae of the fungus gnat *B. impatiens* that ingested oospores and mycelium of *P. aphanidermatum* and then fed on cucumber roots growing in rockwool apparently introduced the pathogen to these plants (21).

Although our studies demonstrate that previous exposure of geranium seedlings to fungus gnat larvae can result in reduced levels of *Pythium* infection, the development of new management strategies for fungus gnats is not warranted. Direct root feeding damage by fungus gnat larvae and transmission of plant-pathogenic conidial fungi by adult fungus gnats is well

documented; thus, these insects should still be considered pests in most contexts (12, 19, 23). Furthermore, the mechanisms of the observed interaction may be complex, with various additional factors affecting expression in commercial crop production systems, and investigation of these mechanisms is warranted. Overall, the findings of this study enhance our understanding of the association between fungus gnats and *Pythium* in greenhouse floriculture and indicate that ecological studies are needed to thoroughly explore soil pathosystems.

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CHAPTER 6: GENERAL SUMMARY

The primary objective of these dissertation studies was to elucidate the role played by fungus gnats, *Bradysia impatiens*, in the transmission of root rot diseases of floriculture crops. Although there is evidence that adult fungus gnats can vector aerial-spore-producing fungal pathogens, including *Fusarium*, *Thielaviopsis*, and *Verticillium* spp. (1, 2, 3, 5, 7, 10), a significant role in the transmission of pathogens that do no generate large numbers of readily transmissible propagules has not been demonstrated. Despite evidence of low potential for vectoring pathogens of this type such as *Pythium* in the class Oomycetes by adult fungus gnats (4, 9), the perception has persisted for decades that these insects are important vectors of *Pythium* spp. (8, 12).

To elucidate the role of fungus gnats in the possible transmission of *Pythium* spp. root rots, laboratory studies were conducted to address the following objectives: 1) What are larval and adult fungus gnat attraction and oviposition responses to plants infected/infested with *Pythium* spp. (as well as several other microorganisms) versus non-infected/infested plants? 2) What is the potential of adult fungus gnats to acquire infectious propagules of *Pythium* spp. from diseased plants and transmit them to healthy plants under laboratory conditions highly favorable for infection? 3) Can natural variation in the biology of *Pythium* strains affect the likelihood of transmission by fungus gnats? 4) Does wounding damage inflicted by fungus gnats affect the susceptibility of geranium seedlings by *Pythium aphanidermatum*?

In general, very little is known about the capacity of fungus gnats to utilize microorganisms other than true fungi. The close association between fungus gnats and diseased plants may be based primarily on a fungus gnat strong attraction to some aspect of microbial activity in diseased plant tissues. If so, fungus gnats thus may become associated with plants

infected by microbes that they may or may not vector. Laboratory tests were conducted to determine the preferences of larval and adult fungus gnats for a variety of microbes associated with greenhouse crops. Geranium seedlings were found to be particularly vulnerable to infection by *Pythium* spp. and were used in all experiments in this dissertation. Fungus gnat adult females strongly preferred to oviposit on plant material infected/infested with a wide array of microorganisms, such as several species of *Pythium*, including non-pathogenic species, the pathogenic fungus *Thielaviopsis basicola*, the pathogenic bacterium *Xanthomonas campestris* pv. *pelargonii*, the beneficial pathogen-suppressive fungus *Trichoderma harzianum*, and the insect-pathogenic fungus *Beauveria bassiana*. Larvae were also attracted to various *Pythium* species. In fact, fungus gnats were attracted to every microorganism tested, regardless of whether they are suspected vectors (such as in the case of *Xanthomonas*). Only if the microorganisms were killed were the fungus gnats no longer attracted to them. These findings emphasize the importance of sanitizing greenhouses to minimize unnecessary microbial activity as part of fungus gnat control. Also, if fungus gnat adults are attracted to microbial activity, perhaps it may be possible to attract them away from crop plants by providing traps that have high microbial activity.

Although determining that fungus gnats are stimulated to oviposit by a wide array of living microorganisms helped explain the associated of fungus gnats with diseased plants, the purported role of fungus gnats in the epidemiology of *Pythium* spp. was still unclear. A series of laboratory experiments were conducted to determine whether fungus gnat adults can be vectors of several common greenhouse *Pythium* spp., including *P. aphanidermatum*, *P. irregulare*, and *P. ultimum*. An additional experiment sought to determine whether *P. aphanidermatum* can be maintained transstadially in the gut of a fungus gnat larva to be transmitted by the subsequent adult. Several different experimental approaches consistently failed to show that adult fungus

gnats would acquire and transmit infectious *Pythium* propagules from diseased to healthy plants. A molecular assay (primers and probe for real-time polymerase chain reaction) was developed for species-specific detection and quantification of *P. aphanidermatum*. Use of the molecular assay demonstrated that *Pythium* ingested by fungus gnat larvae is not carried beyond the pupal stage. These results from this series of experiments demonstrate that adult fungus gnats are unlikely crop-to-crop or greenhouse-to-greenhouse vectors of *Pythium* root rot pathogens.

Though it appeared that adults are unlikely vectors of *Pythium*, the possible role of the larvae was still not clear. A series of laboratory experiments were conducted to investigate the *Pythium* spp.–vectoring potential of larval fungus gnats by assessing the capacity of larvae to ingest propagules from two strains each of *P. aphanidermatum* and *P. ultimum* and transmit the pathogens to healthy geranium seedlings under laboratory conditions. This final study was also undertaken to examine some observed morphological differences in various strains of *Pythium* spp. and the potential variation in larval fungus gnat vectoring potential of *Pythium* based on pathogen species and strain. Assays revealed that the *Pythium*–vectoring potential of larval fungus gnats varies greatly by species and strain and that passage of certain strains through larval guts significantly increases the oospore germination rate and vectoring potential. Although these results are interesting from mycological and plant disease perspectives, it is unlikely that these slow-moving larvae account for significant transmission of *Pythium* spp. between greenhouse facilities or even between plant pots on a greenhouse bench. Rarely, if ever, do fungus gnat larvae move between plants growing in separate greenhouse containers (J. Sanderson, pers. obs.), though plant-to-plant movement may occur in a continuous growing substrate, such as rockwool slabs used in cucumber or tomato production. Also, *Pythium* spp. are fully capable of persisting in the soil or other plant growing media and dispersing short distances through soil or other

media as swimming zoospores or by growing mycelia without the reliance on fungus gnat larvae for transport.

Previous studies on the effects of larval fungus gnats on plants have shown that their feeding may promote plant infection by pathogenic fungi (6, 11), whereas other studies have demonstrated no enhanced infection (5). Laboratory tests were conducted to investigate the potential effects of fungus gnat feeding damage on the susceptibility of geranium seedlings to infection by the root rot pathogen *P. aphanidermatum*. My experiments demonstrated a pronounced negative fungus gnat–*Pythium* interaction, with exposure to fungus gnats prior to inoculation with *P. aphanidermatum* resulting in significantly fewer seedling deaths than would have been expected if the two agents had acted independently. Similar results were found when seedlings were subjected to mechanical wounding prior to *Pythium* inoculation. The degree of plant damage inflicted by larval fungus gnat feeding did not have a significant effect on the combined damage from fungus gnat larvae and *Pythium*. These results suggest that low-level damage by fungus gnats may actually benefit host plants by stimulating defenses against disease-causing microbes.

In general, the studies conducted in this dissertation provide a detailed understanding of the association between fungus gnats and *Pythium* spp. on plant roots in greenhouse floriculture. It appears that the observed association between fungus gnats and diseased plants is based primarily on a strong attraction to microbial activity in diseased plant tissues. But adult fungus gnats are unlikely vectors of *Pythium* spp. However, relaxing management strategies for fungus gnat control is not warranted. Fungus gnats should still be considered pests in most cases because they are capable of directly wounding plants and transmitting several plant-pathogenic conidial fungi.

Future disease epidemiology studies with fungus gnat should focus on several questions: 1) the mechanism or mechanisms underlying the finding that larval fungus gnat feeding inhibits *Pythium* infection of geranium seedlings; 2) factors mediating ovipositional substrate selection by fungus gnats; 3) physiological processes associated with the degradation of ingested *Pythium* propagules during eclosion from the fungus gnat pupal to adult stage; and 4) the development of traps that have high microbial activity to attract fungus gnat adults away from crop plants.

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