

Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution

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Abstract Dual biological control, of both insect pests and plant pathogens, has been reported for the fungal entomopathogens, *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales) and *Lecanicillium* spp. (Ascomycota: Hypocreales). However, the primary mechanisms of plant disease suppression are different for these fungi. *Beauveria* spp. produce an array of bioactive metabolites, and have been reported to limit growth of fungal plant pathogens in vitro. In plant assays, *B. bassiana* has been reported to reduce diseases caused by soilborne plant pathogens, such as *Pythium*, *Rhizoctonia*, and *Fusarium*. Evidence has accumulated that *B. bassiana* can endophytically colonize a wide array of plant species, both monocots and dicots. *B. bassiana* also induced systemic

resistance when endophytically colonized cotton seedlings were challenged with a bacterial plant pathogen on foliage. Species of *Lecanicillium* are known to reduce disease caused by powdery mildew as well as various rust fungi. Endophytic colonization has been reported for *Lecanicillium* spp., and it has been suggested that induced systemic resistance may be active against powdery mildew. However, mycoparasitism is the primary mechanism employed by *Lecanicillium* spp. against plant pathogens. Comparisons of *Beauveria* and *Lecanicillium* are made with *Trichoderma*, a fungus used for biological control of plant pathogens and insects. For *T. harzianum* Rifai (Ascomycota: Hypocreales), it has been shown that some fungal traits that are important for insect pathogenicity are also involved in biocontrol of phytopathogens.

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Introduction

Resource availability can trigger shifts in functionality within a fungal species, thereby changing the ecological role of the organism (Termorshuizen and Jeger 2009). Shifts from one resource to another may necessitate significant adaptations in metabolism, particularly if the resources are dissimilar (Leger

et al. 1997). Among members of the Hypocreales, animal, fungal, and plant resources are exploited. These fungi gain nutrition in a variety of ways, including: saprotrophs that colonize the rhizosphere and phyllosphere, endophytic saprotrophs, hemibiotrophs and necrotrophs of plants, entomopathogens, and mycoparasites. Some of these fungi function in more than one econutritional mode. Fungi traditionally known for their entomopathogenic characteristics, such as *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales) and *Lecanicillium* spp. (Ascomycota: Hypocreales), have recently been shown to engage in plant-fungus interactions (Vega 2008; Vega et al. 2008), and both have been reported to effectively suppress plant disease (Goettel et al. 2008; Ownley et al. 2008).

Mechanisms of plant disease suppression by biocontrol fungi

Biological control of plant pathogens usually refers to the use of microorganisms that reduce the disease-causing activity or survival of plant pathogens. Several different biological control mechanisms against plant pathogens have been identified. With some mechanisms, such as antibiosis, competition, and parasitism, the biocontrol organism is directly involved. With other modes of biological control, such as induced systemic resistance and increased growth response, endophytic colonization by the biocontrol organism triggers responses in the plant that reduce or alleviate plant disease.

Antibiosis, competition, and mycoparasitism

The mechanism of antibiosis includes production of antibiotics, bioactive volatile organic compounds (VOCs), and enzymes. Volatile bioactive compounds include acids, alcohols, alkyl pyrones, ammonia, esters, hydrogen cyanide, ketones, and lipids (Ownley and Windham 2007). The fungal endophyte *Muscodor albus* Worapong, Strobel & W.M. Hess (Ascomycota: Xylariales) produces a mixture of VOCs that are lethal to a variety of microorganisms (Strobel et al. 2001; Mercier and Jiménez 2004; Mercier and Smilanick 2005; Strobel 2006), as well as to insects (Riga et al. 2008; Lacey et al. 2009). In the first report of VOCs

released by a fungal entomopathogen, carbon source played a major role in VOC production by *B. bassiana*. When cultured on glucose-based media, the VOCs identified were diisopropyl naphthalenes (<50%), ethanol (ca. 10%) and sesquiterpenes (6%), but in media with n-octacosane (an insect-like alkane), the primary VOCs were *n*-decane (84%) and sesquiterpenes (15%) (Crespo et al. 2008).

Enzymes involved in antibiosis are distinctly different from those involved in mycoparasitism of plant pathogens. For example, the biocontrol fungus *Talaromyces flavus* Tf1 (Klöcker) Stolk & Samson (Ascomycota: Eurotiales) produces the enzyme glucose oxidase, whose reaction product, hydrogen peroxide, kills microsclerotia of phytopathogenic *Verticillium* (Fravel 1988).

Fungal biocontrol organisms actively compete against plant pathogens for niche or infection site, carbon, nitrogen, and various microelements. The site of competition is often the rhizosphere, phyllosphere, or intercellularly within the plant. Successful competition is often a matter of timing as resources are likely to go to the initial colonizer.

Mycoparasitism is the parasitism of one fungus by another. Varying degrees of host specificity are displayed by mycoparasites. Within a given species of mycoparasite, some isolates may infect a large number of taxonomically diverse fungi, while others demonstrate a high level of specificity (Askary et al. 1998). As reviewed in Harmon et al. (2004), parasitism by the biocontrol fungus *Trichoderma* (Ascomycota: Hypocreales) begins with detection of the fungal host before contact is made. *Trichoderma* produces low levels of an extracellular exochitinase, which diffuse and catalyze the release of cell-wall oligomers from the target host fungus. This activity induces *Trichoderma* to release fungitoxic endochitinases, which also degrade the fungal host cell wall. Attachment of the mycoparasite to the host fungus is mediated by binding of carbohydrates in the *Trichoderma* cell wall to lectins in the cell wall of the fungal host. Upon contact, hyphae of *Trichoderma* coil around the host fungus and form appressoria. Several lytic enzymes are involved in degradation of the cell walls of fungal and oomycetous plant pathogens, including chitinases, β -1,3 gluconases, proteases, and lipases.

In many cases, mechanisms of biocontrol are not mutually exclusive, i.e. multiple mechanisms may be

operating against a specific plant pathogen, or a given biocontrol fungus may employ different mechanisms against different phytopathogens. For example, control of *Botrytis cinerea* Pers. (Ascomycota: Helotiales) on grapes (*Vitis*) with *Trichoderma* involves competition for nutrients and mycoparasitism of sclerotia, the overwintering, long-term survival structure of *Botrytis*. Both mechanisms contribute to suppression of the pathogen's capability to cause and perpetuate disease (Dubos 1987). Following application to leaves as a preventative, *Trichoderma* induced resistance to downy mildew, *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni (Oomycota: Peronosporales), in grape (Perazzolli et al. 2008). Therefore, it is possible that induced systemic resistance may also play a role in biocontrol of *Botrytis*. Induced resistance to *Botrytis*, following application of *T. harzianum* T39 Rifai (Ascomycota: Hypocreales) to roots and leaves of several ecotypes of *Arabidopsis thaliana* (L.) Heynh. has been reported (Korolev et al. 2008).

Induced systemic resistance

Plants are sessile organisms that must develop a complex chemical arsenal in order to withstand biotic and abiotic attack. Colonization of plants with nonpathogenic fungi and bacteria can lead to induced systemic resistance (ISR) in the host plant. Induced resistance is a plant-mediated biocontrol mechanism whereby the biocontrol agent and the phytopathogen do not make physical contact with one another. Plants react to the presence of a pathogen with a rapid expression of defense-related genes. For example, dramatic cellular changes, characterized by rapid necrotization of lemon (*Citrus × limon* (L.) Burm. f.) fruit exocarp cells were observed in fruit treated with *Lecanicillium muscarium* DAOM 198499 (Petch) Zare & W. Gams (formerly *Cephalosporium muscarium* Petch). Phenolic compounds and phenol oxidase were both present in reactive cells (Benhamou 2004).

In contrast, gene expression changes in plants infected with beneficial fungi tend to be mild, and the relationship is allowed to develop resulting in an infected or colonized plant. The signaling mechanisms for this induced resistance are based on jasmonic acid (JA) and ethylene (Van Loon et al. 1998; Van Wees et al. 2008; Gutjahr and Paszkowski

2009). Induction of systemic resistance via the JA/ethylene signaling pathway has been reported primarily for plant growth-promoting bacteria, however, it is also operative for many mycorrhizal fungi (Gutjahr and Paszkowski 2009) and biocontrol fungi (Harmon et al. 2004; Vinale et al. 2008).

Endophytism by fungal entomopathogens

Even though the term “endophyte” has several definitions (Hyde and Soyong 2008), it is widely accepted that endophytes are microorganisms present in plant tissues without causing any apparent symptoms. Fungal endophytes are widespread and quite diverse in nature (Arnold et al. 2000; Arnold 2007). For example, Vega et al. (2009b) reported 257 unique ITS genotypes for fungal endophytes isolated from coffee plants in Hawaii, Mexico, Colombia, and Puerto Rico. Infection by fungal endophytes can be localized (i.e., not systemic; see Saikkonen et al. 1998 and references therein), and establishing a long-term systemic infection with endophytic fungal entomopathogens that can act against plant pathogens will remain a challenge, and should be the focus of intensive study.

Isolation of *B. bassiana* as a fungal endophyte has been reported for many plants under natural conditions, as well as in plants inoculated using various methods (Vega 2008; Vega et al. 2008). In contrast to the several studies dealing with endophytic *Beauveria* spp., only a handful of studies have been conducted on endophytic *Lecanicillium* spp. For example, *Lecanicillium dimorphum* (J.D. Chen) Zare & W. Gams and *L. cf. psalliotae* (Treschew) Zare & W. Gams have been introduced as endophytes in date palms (*Phoenix dactylifera* L.) (Gómez-Vidal et al. 2006), and *L. muscarium* strain DAOM 198499 (= *Verticillium lecanii* (Zimm.) Viégas) and *L. muscarium* strain B-2 have been introduced as endophytes in cucumber (*Cucumis sativus* L.) roots (Benhamou and Brodeur 2001; Hirano et al. 2008). In cytological investigations of cucumber roots, the entomopathogen grew actively at the root surface and colonized a small number of epidermal and cortical cells, without inducing extensive host cell damage. Ingress into the root tissue was primarily intercellular and cell wall penetration was seldom observed (Benhamou and Brodeur 2001). *Verticillium*

(=*Lecanicillium*) *lecanii* has been reported as a natural endophyte in an Araceae (Pettrini 1981), in *Arctostaphylos uva-ursi* (L.) (Widler and Müller 1984), and in *Carpinus caroliniana* Walter (Bills and Polishook 1991).

Although traditionally categorized as a soil saprophyte, *Beauveria* spp. are considered to be poor competitors for organic resources against other ubiquitous saprophytic soil fungi (Keller and Zimmermann 1989; Hajek 1997). The endophytic habit of *B. bassiana* may provide benefits to both plant and fungus. It is well known that plant species has a significant impact on shaping plant-associated microbial communities (Berg et al. 2005; reviewed in Berg and Smalla 2009). As suggested by the bodyguard hypothesis, the plant gains through reduction of damage against herbivorous insects (Elliot et al. 2000; White et al. 2002) or plant diseases; the fungus benefits through protection from environmental stress, acquisition of limited nutrients from endophytic colonization as well as exudates on the plant surface, and use of the plant surface as a staging platform for insect parasitism. On tomato (*Solanum lycopersicum* L.) and other dicots, as well as monocots, colonization by *B. bassiana* is not restricted to growth as an endophyte (Ownley et al. 2008; Powell et al. 2009; authors, unpublished data). From initial establishment as a seed treatment, the fungus can be found on the outer surfaces as the plant ages, particularly in areas where new leaves or shoots have emerged. The fungus also gains from nutrients acquired during saprophytic colonization of the plant when it, or parts of it senesce. Similar epiphytic growth was observed by Posada and Vega (2005) with cocoa (*Theobroma cacao* L.) seedlings.

***Beauveria bassiana*: Potential for biological control of plant pathogens**

Beauveria bassiana is known to occur naturally in more than 700 species of insect hosts (Inglis et al. 2001). Infection of host insects results in the production of large numbers of conidia, thereby serving to increase the population size of the fungus (Meyling and Eilenberg 2007). There is now substantial evidence that *B. bassiana* can provide protection against some soilborne plant pathogens (Ownley et al. 2004; Ownley et al. 2008; Vega et al.

2009a, b). It is likely that more than one mode of action is operative in suppression of plant disease by *B. bassiana*. Isolates of the fungus are known to produce numerous secondary metabolites (e.g. beauvericin, beauverolides, bassianolides, oosporein, cyclosporin A, and oxalic acid) with antibacterial, antifungal, cytotoxic, and insecticidal activities (Grove and Pople 1980; Genthner et al. 1994; Gupta et al. 1995; Boucias and Pendland 1998; Copping and Menn 2000). Effects of these compounds on microorganisms and insects have been reported (Kanaoka et al. 1978; Taniguchi et al. 1984; Eyal et al. 1994; Boucias et al. 1995). Recently, another antimicrobial compound, bassianolone, from *B. bassiana* fermentation culture under low nitrogen conditions, was characterized (Oller-López et al. 2005). Bassianolone has activity against fungi and Gram-positive cocci. Antibiosis assays with *B. bassiana* against various plant pathogens in vitro have been reported (Table 1). However, the antimicrobial compounds were not identified.

Beauveria bassiana strain 11-98 suppresses plant disease caused by the soilborne plant pathogens *Rhizoctonia solani* Kühn (Basidiomycota: Cantharellales) (Ownley et al. 2004) and *Pythium myriotylum* Drechsler (Oomycota: Pythiales) (Clark et al. 2006). This isolate produces beauvericin (Leckie et al. 2008) and oosporein (authors, unpublished data), but it is not known if these compounds play a role in suppression of plant disease. Biological control of plant pathogens with *B. bassiana* 11-98 is likely to involve competition for resources (Ownley et al. 2004), since the fungus is a plant colonist. Application of *B. bassiana* 11-98 to tomato seed resulted in endophytic and epiphytic colonization of seedlings and subsequent protection against damping-off. Similarly, seed treatment of cotton (*Gossypium hirsutum* L.) reduced severity of *R. solani* damping-off in seedlings (Griffin 2007; Ownley et al. 2008). In both tomato and cotton, the degree of disease control achieved with *Beauveria bassiana* was correlated with the population density of conidia established on seed (Ownley et al. 2008; authors, unpublished data). Smaller seeds, such as tomato were protected more effectively with rates of 1×10^6 – 10^7 CFU/seed, while higher rates (1×10^7 – 10^9 CFU/seed) gave the greatest protection against seedling disease in cotton.

Parasitism of *Pythium myriotylum* by *B. bassiana* may be involved in suppression of *Pythium* damping-

Table 1 Studies reporting activity of *Beauveria* spp. against plant pathogens

Strain or species of <i>Beauveria</i>	Type of study	Plant pathogen	Activity against plant pathogen	Reference
<i>Beauveria bassiana</i> , isolated from wheat rhizosphere	In vitro bioassay <i>In planta</i> (wheat), pot assays	<i>Gaeumannomyces graminis</i> var. <i>tritici</i> J. Walker (Ascomycota: Sordariomycetidae)	Inhibited growth; produced chitinase and β -glucanases Suppressed take-all disease	Renwick et al. (1991)
<i>Beauveria bassiana</i> (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales), five different isolates	In vitro bioassay	<i>Fusarium oxysporum</i> E.F. Smith & Swingle (Ascomycota: Hypocreales) <i>Armillaria mellea</i> (Vahl) P. Kumm (Basidiomycota: Agaricales) <i>Rosellinia necatrix</i> Berl. ex Prill. (Ascomycota: Xylariales)	All <i>Beauveria</i> isolates inhibited mycelial growth of the pathogens tested	Reisenzein and Tiefenbrunner (1997)
Culture filtrate of <i>B. bassiana</i>	In vitro bioassay	<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i> (Sacc.) W.C. Snyder & H.N. Hansen (Ascomycota Hypocreales) <i>Botrytis cinerea</i> Pers. (Ascomycota: Helotiales)	Inhibited mycelial growth; Inhibited and delayed conidial germination	Bark et al. (1996)
<i>B. bassiana</i>	In vitro bioassay	<i>Pythium ultimum</i> Trow (Oomycota: Pythiales), <i>Pythium debaryanum</i> R. Hesse (Oomycota: Pythiales), <i>Septoria nodorum</i> (= <i>Phaeosphaeria nodorum</i> (E. Müll.) Hedjar. (Ascomycota: Pleosporales)	Caused cell lysis; inhibited mycelial growth Did not inhibit mycelial growth of these pathogens	Vesely and Koubova (1994)
Culture filtrates of <i>Beauveria</i> sp.	In vitro bioassay	<i>Rhizoctonia solani</i> Kühn (Basidiomycota: Cantharellales), <i>Pythium irregular</i> Buisman (Oomycota: Pythiales), <i>Phoma betae</i> (= <i>Pleospora betae</i> Björl. (Ascomycota: Pleosporales)), <i>Phoma exigua</i> var. <i>foveata</i> Malc. & E.G. Gray (Ascomycota: Pleosporales) <i>Rhizoctonia solani</i>	Inhibited mycelial growth; stimulated growth of cucumber	Lee et al. (1999)
<i>B. bassiana</i> 142, applied to onion bulbs	<i>In planta</i> (onion), field and greenhouse	<i>Fusarium oxysporum</i> f. sp. <i>cepae</i> (Hanzawa) W.C. Snyder & H.N. Hansen (Ascomycota: Hypocreales)	Increased bulb germination; reduced plant infection	Flori and Roberti (1993)
<i>B. bassiana</i> 11-98, applied as a seed treatment	<i>In planta</i> (tomato), greenhouse	<i>Rhizoctonia solani</i>	Reduced damping off of seedlings; increased plant growth	Ownley et al. (2000) and Ownley et al. (2004)
<i>B. bassiana</i> 11-98, applied as a seed treatment	<i>In planta</i> (tomato), growth chamber	<i>Pythium myriophyllum</i> Drechsler (Oomycota: Pythiales)	Reduced damping off of seedlings	Clark et al. (2006)
<i>B. bassiana</i> 11-98	In vitro bioassay	<i>Rhizoctonia solani</i>	Did not inhibit mycelial growth of <i>R. solani</i> ; but hyphae of 11-98 coiled around	Griffin (2007)
<i>B. bassiana</i> 11-98, applied as a seed treatment	<i>In planta</i> (cotton), growth chamber	<i>Pythium myriophyllum</i>	hyphae of <i>P. myriophyllum</i> , which suggested parasitism Reduced damping-off of seedlings	and Ownley et al. (2008)

off in tomato seedlings. In dual culture, hyphae of isolate 11-98 were observed coiling around the larger coenocytic hyphae of *P. myriotylum* (Griffin 2007).

The extent of endophytic colonization of tomato by *B. bassiana* 11-98 was also correlated with the rate of conidia applied to seed. Rates that were most effective in disease control also resulted in the greatest degree of plant colonization. *Beauveria bassiana* was detected in root, stem, and leaf sections of surface-sterilized tomato seedlings with standard dilution plating procedures onto semi-selective medium (Ownley et al. 2008). In addition to seedlings, *B. bassiana* 11-98 has been recovered from foliage, stem, and root tissues of surface-sterilized 18-week-old tomato plants produced from treated seed (Powell et al. 2009). *Beauveria bassiana* has also been recovered as an endophyte of eastern purple coneflower (*Echinacea purpurea* L. Moench), cotton, snap bean (*Phaseolus vulgaris* L.), soybean (*Glycines max* L.), and switchgrass (*Panicum virgatum* L.) following application of conidia to seed (Griffin 2007; Ownley et al. 2008; authors, unpublished data).

Endophytic *B. bassiana* 11-98 has been observed with scanning electron microscopy (SEM), and detected with polymerase chain reaction (PCR) in cotton seedlings (Griffin 2007). Using SEM on seedlings maintained in a sterile system, conidial germination and hyphal growth were observed in association with areas of leaf exudation. Penetration points through epithelial cells were observed, without formation of a specialized structure. Hyphae ramified through the palisade parenchyma and mesophyll layers of leaf tissues. *Beauveria bassiana* 11-98 was also detected with PCR in a mixed DNA sample of 1 part *B. bassiana* DNA to 1,000 parts cotton DNA, and from surface-sterilized tissues of cotton seedlings grown from *B. bassiana*-treated seed (Griffin 2007; Ownley et al 2008; authors, unpublished data).

The results of a study with cotton seedlings suggested that induced systemic resistance is also a probable mechanism of biological control for *B. bassiana* 11-98 (Griffin 2007; Ownley et al. 2008; authors, unpublished data). Isolate 11-98 was evaluated for its ability to induce systemic resistance in cotton against *Xanthomonas axonopodis* pathovar *malvacearum* (causes bacterial blight). Conidia of *B. bassiana* were applied as a root drench to 5-day old seedlings, 13 days prior to pathogen challenge. Treatment with *B. bassiana* (at 10^7 CFU/seedling

root) resulted in significantly lower foliar disease ratings for bacterial blight than the untreated control and was as effective as 2,6-dichloro-isonicotinic acid, which has been shown to induce systemic resistance against plant pathogens.

***Lecanicillium* spp. and biological control of plant pathogens**

Lecanicillium spp. (formerly classified in the single species *Verticillium lecanii*) are well known as entomopathogens of aphids and scale insects (Hall 1981; Goettel et al. 2008). These fungi are also known as mycoparasites of species of plant pathogenic, biotrophic powdery mildew (Hall 1980; Verhaar et al. 1996) and rust fungi (Spencer and Atkey 1981; Allen 1982; Whipps 1993) on various vegetable, fruit, and ornamental crops, and as pathogens of plant parasitic nematodes (Meyer et al. 1990; Shinya et al. 2008). Activity of *Lecanicillium* spp. against both plant pathogens and insects has been demonstrated in bioassays (Askary et al. 1998; Askary and Yarmand 2007; Kim et al. 2007) and greenhouse studies (Kim et al. 2008) (Table 2).

Commercial products containing *Lecanicillium* spp. have not been developed for plant disease control. However, a formulation of *L. longisporum* (Petch) Zare & W. Gams, known as Vertalec[®], is available for control of insect pests. *Lecanicillium longisporum* (applied as Vertalec[®]), *Lecanicillium attenuatum* Zare & W. Gams CS625, and *Lecanicillium* sp. DAOM 198499 suppressed development of powdery mildew, *Podosphaera fuliginea* (Schldt.) U. Braun & S. Takam. (Ascomycota: Erysiphales) (=synonym *Sphaerotheca fuliginea*) on cucumber leaf discs when applied one or eight days after powdery mildew inoculation. When applied to highly infected leaf discs 11–15 days after pathogen inoculation, *Lecanicillium* treatments significantly suppressed subsequent production of powdery mildew spores, compared to controls (Kim et al. 2007). In greenhouse experiments, *L. longisporum* (applied as Vertalec[®]) suppressed spore production of powdery mildew on potted cucumber plants under conditions of low and high infection levels (Kim et al. 2008).

Askary et al. (1997) provided ultrastructural and cytochemical evidence for the process of parasitism of *P. fuliginea* by *Lecanicillium* sp. DAOM 198499

Table 2 Studies on *Lecanicillium* spp. as dual biological controls for plant pathogens and insect pests

Species or strain of <i>Lecanicillium</i> ^a	Type of study	Plant pathogen	Mode of action against plant pathogen	Insect	Reference
<i>V. lecanii</i> Vertalec DAOM 216596 (see below) DAOM 198499 (see below)	Laboratory bioassay	<i>Podosphaera fuliginea</i> (Schltld.) U. Braun & S. Takam. (Ascomycota: Erysiphales) (syn. <i>Sphaerotheca fuliginea</i>) Powdery mildew	Parasitism/antibiosis	<i>Macrosiphum euphorbiae</i> (Hemiptera: Aphididae)	Askary et al. (1998)
<i>L. muscarium</i> (Petch) Zare & W. Gams (Ascomycota: Hypocreales) strain DAOM 198499	Laboratory bioassay	<i>P. fuliginea</i> (syn. <i>S. fuliginea</i>)	Parasitism	<i>M. euphorbiae</i> <i>Aphidius nigripes</i> (Hymenoptera: Braconidae)	Askary and Yarmand (2007)
<i>L. longisporum</i> (Petch) Zare & W. Gams (Ascomycota: Hypocreales) (Vertalec)	Laboratory bioassay	<i>P. fuliginea</i> (syn. <i>S. fuliginea</i>)	Not reported	<i>Myzus persicae</i> (Hemiptera: Aphididae) <i>M. euphorbiae</i> <i>Aulacorthum solani</i> (Hemiptera: Aphididae)	Kim et al. (2007)
<i>L. attenuatum</i> Zare & W. Gams (Ascomycota: Hypocreales) strain CS625 <i>Lecanicillium</i> sp. strain DAOM 198499					
<i>L. longisporum</i> (Vertalec)	Greenhouse	<i>P. fuliginea</i> (syn. <i>S. fuliginea</i>)	Not reported	<i>Aphis gossypii</i> (Hemiptera: Aphididae)	Kim et al. (2008)
<i>L. lecanii</i> (Zimm.) Zare & W. Gams (Ascomycota: Hypocreales)	Field (survey)	<i>Hemileia vastatrix</i> Berk. & Broome (Basidiomycota: Pucciniales) Coffee leaf rust	Parasitism	<i>Coccus viridis</i> (Hemiptera: Coccidae)	Vandermeer et al. (2009)

^a Name listed is the same as was given in the reference

(formerly *V. lecanii* DAOM 198499), including production of cell-wall degrading enzymes such as chitinases. They suggested that prior to invasion of *P. fuliginea*, the powdery mildew fungus was weakened by antibiotics produced by *Lecanicillium* (Askary et al. 1997). Subsequently, Benhamou and Brodeur (2000) showed that this strain does produce anti-fungal compounds in culture that are effective against *Penicillium digitatum* (Pers.) Sacc. (Ascomycota: Eurotiales), which causes postharvest green mold of citrus. It has been suggested that production of antimicrobial compounds that weaken or kill the target host cells prior to parasitism is a form of specialized saprophytism, rather than parasitism (Bélanger and Labbé 2002).

In most of the studies with *Lecanicillium* as a biological control against plant pathogens, activity

has been attributed to parasitism. Indeed, an array of extracellular lytic enzymes have been reported for isolates of *Lecanicillium*, including cellulases, proteases, β -1,3-glucanases, chitinases (Bidochka et al. 1999; Saksirirat and Hoppe 1991) and more recently, pectinases (Benhamou and Brodeur 2001). However, induction of plant host defense reactions against *P. digitatum* (Benhamou and Brodeur 2000; Benhamou 2004), *Pythium ultimum* Trow (Oomycota: Pythiales) (Benhamou and Brodeur 2001), and powdery mildew (Hirano et al. 2008) have been reported. In studies on biological control of *P. ultimum*, *Lecanicillium* sp. DAOM 198499 grew intercellularly among epidermal and cortical cells on cucumber roots treated with the fungus (Benhamou and Brodeur 2001). Endophytic colonization of cucumber roots was also observed when blastospores of *L. muscarium* B-2

were applied to roots. Subsequently induced resistance to powdery mildew on the cucumber leaf surface was reported (Hirano et al. 2008). Koike et al. (2004) demonstrated that *L. muscarium* B-2 is also a very successful epiphytic colonist of cucumber leaf surfaces, suggesting that competition for nutrients and space may also be operative against powdery mildew.

Fungal endophytism and induced systemic resistance

Recently, proteomic analysis of *P. dactylifera* infected with endophytic *B. bassiana* or two *Lecanicillium* spp. was reported by Gómez-Vidal et al. (2009). Colonization by *B. bassiana*, *L. dimorphum*, or *L. cf. psalliotae* resulted in induction of proteins related to plant defense or stress response, and proteins involved in energy metabolism and photosynthesis were also affected. As additional studies on molecular analysis of plants infected with endophytic fungal entomopathogens are conducted, it will become evident that endophytism is inducing important changes in plant metabolism, even though the plant does not present any symptoms of endophyte infection. It will be important to take into consideration that endophytes may cause plants to enter a “primed state” (*sensu* Conrath et al. 2006; see also Schulz and Boyle 2005), which could be contributing to the antagonistic effects of *B. bassiana* and *Lecanicillium* on plant pathogenic fungi. It is also possible that endophyte infection might result in positive effects such as enhanced plant growth (Ernst et al. 2003; Schulz and Boyle 2005). Plant growth-related variables should be measured in all studies dealing with the introduction of fungal entomopathogens as possible endophytes, as was recently done by Tefera and Vidal (2009) for sorghum plants inoculated with *B. bassiana*, although it will be difficult to elucidate the role of a specific endophyte if others are already present in the plant.

When endophytism results in “primed” plants, subsequent biotic challenge leads to a transitory period of strongly potentiated gene expression that is associated with accelerated defense responses. These responses confer broad-spectrum resistance to pathogens and insects (Van Wees et al. 2008). In this respect, plants colonized by fungal entomopathogens

resemble plants colonized with plant growth-promoting rhizobacteria (Harmon et al. 2004). Much of the research on systemic resistance of plants infected with endophytic beneficial fungi has focused on mycorrhizal fungi (reviewed in Gutjahr and Paszkowski 2009). These obligate fungi live on plant roots and stimulate plant growth and development by increasing nutrient uptake and decreasing disease and insect problems. While plants infected with hypocrealean fungi do not have the complex structures associated with mycorrhizal infection, they can occupy a nutritional niche in or on the plant and develop an active cross talk with their plant hosts that results in induced resistance (Vinale et al. 2008). Induction of plant resistance has been reported for several species of *Trichoderma* (Harmon et al. 2004; Jeger et al. 2009), and mechanisms for induced resistance are beginning to emerge (Segarra et al. 2007; Vinale et al. 2008). Mechanisms for induced resistance by other hypocrealean fungi are scant, but much information on mechanisms of induced resistance obtained from studies with *Trichoderma* can be applied to other fungal entomopathogens.

Many species of *Trichoderma* have been commercially developed for biological control of plant diseases and insects (Harmon et al. 2004; Shakeri and Foster 2007). Some of these isolates induce resistance to plant pathogens (Table 3). Typically, *Trichoderma* is applied to soil or to plant roots grown in co-culture with the fungus. However, some species induce systemic resistance when leaves are treated with *Trichoderma* conidia (Perazzolli et al. 2008; Korolev et al. 2008). Plant hosts in which resistance is induced are taxonomically diverse and include both monocots and dicots. Several recent studies support jasmonate/ethylene signaling as the mechanism for induced systemic resistance (Table 3), further suggesting that the response is similar to that induced by rhizobacteria (reviewed in Harmon et al. 2004). Induced resistance is broad spectrum, and subsequent challenges of the primed plant by taxonomically diverse pathogens (e.g., bacteria, necrotrophic fungi, biotrophic fungi) induce a rapid and intense activation of cellular defense mechanisms somewhat reminiscent of hypersensitive responses.

Species in the genus *Trichoderma* (Ascomycota: Hypocreales) are well known for the production of bioactive metabolites that play a role in the mycoparasitic or entomopathogenic lifestyles of the

Table 3 Recent evidence for involvement of the jasmonate/ethylene pathway in systemic resistance induced by *Trichoderma* species

Species and strain or extract	Plant	Pathogen	Evidence of effects	Efficacy	References
<i>T. asperellum</i> Samuels, Lieckf. & Nirenberg (Ascomycota: Hypocreales) strain T34, (10 ⁷ spores)	<i>Cucumis sativus</i> L. (cucumber)	<i>Pseudomonas syringae</i> pv <i>lachrymans</i>	Significant increase of jasmonic acid (JA), but not salicylic acid (SA) at 1 h, both peaked at 3 h; JA levels not above untreated control after 6 h, SA decreased until 24 h; Significant increase of peroxidase by 6 h	Reduced bacterial colony forming units by ca. 50%	Segarra et al. (2007)
<i>T. harzianum</i> Rifai (Ascomycota: Hypocreales) strain T39	<i>Arabidopsis thaliana</i> (L.) Heynh.	<i>Botrytis cinerea</i> Pers. (Ascomycota: Helotiales)	Col-0 ecotype, and auxin-resistant and SA acid mutants were ISR-inducible; Mutants impaired in ABA, gibberillic acid, or ethylene/JA were not ISR-inducible	Disease severity reduced in Col-0 following either root or leaf application	Korolev et al. (2008)
<i>T. harzianum</i> strain T39	<i>Vitis vinifera</i> L. cv. Pinot Noir (grape)	<i>Plasmopara viticola</i> (Berk. & M.A. Curtis) Berl. & De Toni (Oomycota: Peronosporales)	Timing and persistence differed from BTH which is SA-dependent	Leaf treatment decreased disease severity; Root treatment did not	Perazzolli et al. (2008)
<i>T. virens</i> (J.H. Mill., Giddens & A.A. Foster) Arx (Ascomycota: Hypocreales) strain Gv29-8	<i>Zea mays</i> L. (corn)	<i>Colletotrichum graminicola</i> (= <i>Glomerella graminicola</i> D.J. Politis (Ascomycota: Sordariomycetidae)	Induction of JA and green leaf volatile biosynthetic genes	Reduced lesion area in leaves from endophytic plants	Djonović et al. (2007)

fungus, as well as in the induction of resistance in plant hosts. Elicitors or resistance inducers can be divided into three broad categories: proteins with enzymatic activity, avirulence-like gene products, and low molecular weight compounds released from cell walls (either fungal or plant) as a result of hydrolytic enzymes (e.g., chitinase, glucanase) (Vinale et al. 2008). In several recent studies, various proteins and peptides from *Trichoderma* have been shown to induce host defense responses (Table 4). Volatiles released after treatment with alamethicin, a 20-amino acid polypeptide isolated from *T. viride* Pers., affect the behavior of the parasitoid *Cotesia glomerata* (L.) (Hymenoptera: Braconidae) (Bruinsma et al. 2009). Wasps chose alamethicin-treated plants over nontreated plants, but chose plants on which *Pieris brassicae* (L.) (Lepidoptera: Pieridae) had fed over alamethicin-treated plants.

Sm1, a hydrophobin-like small protein secreted by *Trichoderma virens* (J.H. Mill., Giddens & A.A. Foster) Arx, was the first non-enzymatic

proteinaceous elicitor determined to be involved in induced resistance responses in rice (*Oryza sativa* L.), cotton, and maize (*Zea mays* L.) (Djonović et al. 2006, 2007). Recently a second small hydrophobin-like protein (Epl1) was isolated from *Hypocrea atroviride* (= *Hypocrea atroviridis* Dodd, Lieckf. & Samuels (Ascomycota: Hypocreales)) (teleomorph of *T. atroviride* P. Karst.) (Vargas et al. 2008). Epl1 was produced as a dimer. Sm1 can also be a dimer, but upon dimerization, the glycosyl moiety and activity are lost. Both hydrophobins are active as resistance inducers when configured as a monomer. Vargas et al. (2008) have proposed that aggregation of the elicitor disrupts the molecular cross-talk between the beneficial fungal colonizer and plant.

Recent proteomic studies provide a glimpse into the complexity of the *Trichoderma*-plant interaction. In cucumber, 51 proteins were different in treatments with *T. asperellum* Samuels, Lieckf. & Nirenberg and untreated controls; 17 proteins were up-regulated, and 11 were down-regulated. Proteins were divided

Table 4 Effects of selected *Trichoderma*-derived peptides and proteins on host defense responses

Peptide/protein	Plant	Effects and efficacy	Reference	Similar compounds described for <i>Beauveria</i> or <i>Lecanicillium</i> spp.
Alamethicin: Ion channel-forming peptide mixture	<i>Brassica oleracea</i> L. var. <i>gemmifera</i> DC. 'Cyrus' (brussel sprouts)	20-fold more potent inducer of ISR than JA; volatile emissions; increased preference for parasitoid wasps (<i>Cotesia glomerata</i> L.) (Hymenoptera: Braconidae)	Bruinsma et al. (2009)	
Mitogen-activated protein kinase TMK1: Serine-threonine kinases	Suspension cells of <i>Arabidopsis thaliana</i> (L.) Heynh. (Col-1) and <i>Nicotiana tabacum</i> L. 'BY-2' (tobacco) <i>Phaseolus vulgaris</i> L. (var. nanus L.) (bean)	Activation of callose synthase; callose deposition Deletion tmk1 mutants had reduced mycoparasitism and host-specific regulation of <i>ech42</i> gene transcription; deletion mutants had an increased ability to protect plants against <i>Rhizoctonia solani</i> Kühn (Basidiomycota: Cantharellales)	Aidemark et al. (2009) Reithner et al. (2007)	Zhang et al. (2009)— <i>Beauveria</i> —regulation of environmental stress and virulence to insects
Sm1: Cerato-platanin protein that is hydrophobin-like	<i>Zea mays</i> L. (corn)	Deletion or over-expression of Sm1 in mutants did not affect normal growth and development of <i>Trichoderma virens</i> (J.H. Mill., Giddens & A.A. Foster) Arx (Ascomycota: Hypocreales); Root colonization was not affected in mutants, but ability to induce resistance to a foliar pathogen was reduced in deletion mutants and increased in some over-expression mutants	Djonović et al. (2007)	Ying and Feng (2004) <i>Beauveria</i> —relationship between hydrophobins and thermotolerance Kamp (2002) <i>Lecanicillium</i> —Hydrophobins abundant in sporulating cultures, but not in mycelial cultures
Ethylene-inducing xyylanase: 18 Kd protein similar to serine protease	<i>Oryza sativa</i> L. 'M-202' (rice); <i>Gossypium hirsutum</i> L. 'Paymaster 2326BG/RR' and 'DeltaPine 50' (cotton) <i>Gossypium hirsutum</i> 'DeltaPine 50' (cotton)	Induced expression of defense genes (glucanase, chitinase) locally and systemically; H ₂ O ₂ produced in Sm1-treated levels, but no resulting necrosis The 18 Kd protein increased terpenoid production and peroxidase activity	Djonović et al. (2006) Hanson and Howell (2004)	
ThPG1 endopolygalacturonase: Cell-wall degrading enzyme associated with pectin degradation	<i>Lycopersicon esculentum</i> (= <i>Solanum lycopersicon</i> L. var. <i>lycopersicon</i>) 'Marmande' (tomato)	ThPG1-silenced mutants had lower polygalacturonase activity and less growth on pectin medium; protection against <i>Botrytis cinerea</i> Pers. (Ascomycota: Helotiales) was the same for ThPG1-silenced mutants and wild type, even though root colonization by mutants was lower	Morán-Díez et al. (2009)	Fenice et al. (1997) <i>Lecanicillium</i> —Antarctic strains of <i>V. (=Lecanicillium) lecanii</i> had wide enzymatic competence, including polygalacturonase activity
ABC transporter membrane pump: ATP-binding cassette with transmembrane domain	<i>L. esculentum</i>	Gene up-regulated in fungus by pathogen-secreted metabolites and some fungicides; deletion mutants were sensitive to fungicides and lost ability to protect against <i>Pythium ultimum</i> Trow (Oomycota: Pythiales) and <i>R. solani</i>	Ruocco et al. (2009)	

into four categories: stress and defense, energy and metabolism, secondary metabolism, and protein synthesis/folding (Segarra et al. 2007). In maize, 114 proteins were up-regulated and 50 were down-regulated in response to treatment with *T. harzianum*. Most of the upregulated genes were for proteins involved in carbohydrate metabolism, defense, and photosynthesis (Shoresh and Harman 2008).

There are several parallels between *Trichoderma* and *Beauveria* and/or *Lecanicillium* spp. that suggest similar mechanisms of induced resistance:

1. These fungi can live endophytically between plant cells without causing negative effects on plant growth and development. Genes with similar function (e.g., plant defense/stress response, energy metabolism, and photosynthesis) are up-regulated in plants colonized by *Beauveria* and *Lecanicillium* (Gómez-Vidal et al. 2009) and those colonized by *Trichoderma* spp. (Segarra et al. 2007; Shoresh and Harman 2008).
2. Plant colonization can be established horizontally by application of spores to seed, roots, or leaves. Even though the relationship between the fungi and their hosts is intimate, plants can easily be infected. This is similar to mycorrhizae but contrasts markedly with the grass endophytes in the genus *Neotyphodium* (Ascomycota: Hypocreales), which are transmitted vertically via seed (Giménez et al. 2007; Hartley and Gange 2009).
3. *Beauveria* and *Trichoderma* spp. are natural and introduced colonists of a wide variety of plants that include both dicots and monocots. Although there is less information available on the plant host range of *Lecanicillium* spp., it has also been recovered as a natural and introduced endophyte of monocots and dicots.
4. All three fungi produce a wide array of enzymes and avirulence-like products. Hydrolytic enzymes that can attack substrates as diverse as plant cell walls, insect cuticle, and oomycetous and fungal plant pathogens are important for the varied nutritional niches occupied by these fungi.
5. *Beauveria bassiana* and many species of *Trichoderma* produce hydrophobins or hydrophobin-like molecules. It has been suggested that the functions of hydrophobins in the life cycle of fungi include: formation of protective layers, attachment, structural components of cell walls, and reduction of surface tension to allow aerial growth (Linder 2009). Hydrophobins produced by *B. bassiana* have been shown to be important in conidial thermotolerance (Ying and Feng 2004) and attachment to substrates (Holder and Keyhani 2005). Hydrophobins of *T. asperellum* were proposed to protect hyphae from defense compounds during the early stages of infection (Viterbo and Chet 2006). Therefore, it is possible that they play a similar role in *B. bassiana*. Hydrophobins have been detected in *Lecanicillium* (Kamp 2002), but little is known on their role in the fungal life cycle.
6. Mitogen-associated protein kinases (MAP kinases) in the subfamily HOG-1 (High osmolarity glycerol (1)) are associated with host infection and with protection from osmotic stress in *Beauveria* and *Trichoderma* spp. The MAP kinases interfere with the ability of *T. atroviride* to induce resistance to the soilborne plant pathogen, *R. solani*, in bean plants. Deletion mutants had a greater ability than wild type to protect the plants. In *B. bassiana*, MAP kinases regulated response of the fungus to stress. Deletion mutants were more sensitive to hyperosmotic stress, high temperature, and oxidative stress than the wild type (Zhang et al. 2009). When transcript levels of hydrophobin-encoding genes in the deletion mutants were low, conidial attachment to cicada hind wings was severely impaired (Zhang et al. 2009).
7. Both *Beauveria* and *Trichoderma* spp. can induce systemic resistance to bacterial pathogens. In cucumber, plants infected by *T. asperellum* (10^7 conidia ml⁻¹) supported less than 50% the number of colony-forming units (CFU) after challenge with *Pseudomonas syringae* pathovar *lachrymans* (Segarra et al. 2007). Treatment of cotton with 1×10^7 CFU *B. bassiana* 11-98 per root induced systemic resistance against bacterial blight (*Xanthomonas axonopodis* pathovar *malvacearum*) on cotton foliage. Although bacterial populations were not assessed, foliar disease ratings were significantly lower for *Beauveria*-treated plants than the untreated control (Griffin 2007).
8. Both *Lecanicillium* and *Trichoderma* spp. can induce systemic resistance to oomycetous plant pathogens. Host plant signaling and subsequent

intense defense responses have been proposed for *Lecanicillium*-treated cucumber. Ingress of *P. ultimum* into roots resulted in the deposition of an electron-opaque material that frequently encircled pathogen hyphae and accumulated in uninfected xylem vessels (Benhamou and Brodeur 2001). Inoculation of roots with *L. muscarium* resulted in root colonization and endophytic growth. Plant leaves were protected from powdery mildew, but defense enzymes were not different in colonized and non-colonized plants (Hirano et al. 2008). *Trichoderma harzianum* induced systemic resistance in pepper plants grown from seed treated with *T. harzianum* spores (Ahmed et al. 2000). Stem lesions, caused by inoculation with *Phytophthora capsici* Leonian (Oomycota: Peronosporales), were 40% shorter than lesions in inoculated plants grown from non-treated seed. *P. capsici* was isolated from zones immediately contiguous with the necrotic tissue, but *T. harzianum* was not, suggesting that there was no direct contact between them. The percentage of *P. capsici* isolated nine days after inoculation was greater in non-treated inoculated plants than in *Trichoderma*-treated plants inoculated with *P. capsici*. In addition to induced resistance against *P. capsici* in the upper part of the plant, concentration of the phytoalexin capsidiol was more than 7-fold greater than in non-treated plants inoculated with *P. capsici*, six days after inoculation (Ahmed et al. 2000).

Conclusions

The ability of many hypocrealean entomopathogens to occupy nutritional niches as diverse as insects, fungi, and plants provides unique opportunities for biological control of multiple plant pathogens and insect pests. Use of these fungi may overcome some of the challenges faced in plant disease control. For example, many foliar phytopathogens have a very high sporulation rate and are well-suited for widespread dissemination as air-borne propagules. If genetic resistance is not available in the crop, fungicide applications are often the primary means of disease control. The rapid reproduction rate of foliar pathogens coupled with frequent applications of systemic fungicides, many of which are narrow spectrum, increases the chances of developing

fungicide resistance in these pathogens (Fry 1982). The ability of the hypocrealean fungi to use several strategies reduces the probability of development of resistance. For example, treatment of roots or seeds with *Beauveria* or *Lecanicillium* spp. conidia potentially produces endophyte-infected plants that reduce initial establishment of the disease through induced resistance. Studies have shown that both *Beauveria* and *Lecanicillium* spp. can become established as epiphytes, which provides opportunities for plant disease suppression through antibiosis, competition, or mycoparasitism. Endophytic and epiphytic populations of these fungi could also reduce insect damage to the plant.

Plant diseases caused by soilborne fungi are notoriously difficult to control since these fungi generally have wide host ranges and can survive in soil for long periods of time as saprophytes or as specialized survival structures (e.g., sclerotia, chlamydospores). Resistant cultivars are available for a limited number of host-pathogen combinations. Soilborne pathogens often cause disease at multiple life stages of the plant (i.e., seed rot, damping-off of seedlings, and root rots), but typically, the greatest impact is on the seed or newly emerged seedling. Use of hypocrealean fungi as plant, seed, or soil treatments facilitates rapid colonization of plant hosts and creates potential for subsequent induced resistance. Older plants may be protected from root rots by induced systemic resistance, although this has not been documented. Seed treatment may also create a potential 'antibiotic' spermosphere that inhibits populations of seed rot pathogens. Mycoparasitism by hypocrealean fungi can be directed against survival structures of soilborne plant pathogens, thus reducing their inoculum potential.

Although much has been accomplished in the commercial development of *Beauveria* and *Lecanicillium* spp. as fungal entomopathogens in plant production, more work is needed to understand the roles of these fungi as epiphytes and endophytes involved in suppression of plant diseases. Some strains of these fungi have been approved for use as bioinsecticides. Use in plant disease control extends development of these products. Future studies should focus on the ecology of these fungi (Vega et al. 2009a, b), their role in plant-microbe interactions, and their antagonism against pathogenic and nontarget microorganisms.

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