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Epizootiology and Phylogenetics of Entomopathogenic Fungi Associated with Fiorinia externa ferris (Hemiptera: Diaspididae) in the Northeastern USA

Jose A. P. Marcelino
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EPIZOOTIOLOGY AND PHYLOGENETICS OF ENTOMOPATHOGENIC FUNGI ASSOCIATED WITH FIORINIA EXTERNA FERRIS (HEMIPTERA: DIASPIDIDAE) IN THE NORTHEASTERN USA

A Dissertation Presented

by

José A. P. Marcelino

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Insect Pathology

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Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, specializing in Plant and Soil Sciences.

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Date: August 24th, 2007
Abstract

The eastern hemlock [*Tsuga canadensis* (L.) Carrière] is one of the native dominant forest components of northeastern US. At present, these valuable stands face an alarming decline, in part due to the *Fiorinia externa*, elongate hemlock scale (EHS), (Hemiptera: Coccoidea: Diaspididae). The armored shield of *F. externa* provides an excellent defense against insecticides, natural enemies and adverse conditions. Chemical and classical biocontrol methods have been unable to stop the spread of this pest. Recently, the occurrence of an epizootic within the *F. externa* population in the Mianus River Gorge Preserve in Bedford, NY revealed a promising opportunity for control of this scale. Entomopathogenic fungi represent a valuable, although under-utilized, group of organisms with unique capabilities for self-sustaining pest management. Given the significant impact of this epizootic on *F. externa*, we have conducted extensive research on the biology, genetics and biological control potential of this epizootic.

We molecularly identified a complex of entomopathogenic, phytopathogenic, and endophytic fungi associated with the epizootic in 36 localities within the states of New York, Pennsylvania, Connecticut and New Jersey. One fungus, *Colletotrichum* sp., was the most commonly isolated organism in populations of *F. externa* within areas of the epizootic. The host range of this *Colletotrichum* species comprised both insects and plants, although diverse life cycles occurred in the different hosts. Endophytic growth was observed in 28 species of plants comprising 18 families (52% of the sampling), whereas in *F. externa* biotrophic and necrotrophic growth was detected. *Colletotrichum* is a widely known phytopathogenic genus and reports of entomopathogenic activity are extremely rare. In order to understand the biological processes involved in the host-pathogen interactions we quantified the pathogenicity and virulence of this *Colletotrichum* sp. to four insect families and six plants families as well as the occurrence of sexual recombination in this *Colletotrichum* sp. both *in vitro* and *in planta*.

We observed that this *Colletotrichum* sp. displays a propensity to induce rapid disease and mortality in *F. externa* hosts. Phylogenetic analysis comprising six of the most commonly studied nuclear genes in molecular phylogenetics (D1/D2 domain of the 28 rDNA gene, ITS region, β-Tubulin 2, GPDH gene, GS gene and HMG box at the MAT1-2 mating-type gene) and RAPDs showed this fungus is closely related to phytopathogenic strains of *Colletotrichum acutatum* and that it may represent a single population lineage of this species (i.e., *Colletotrichum acutatum forma specialis fiorinia*). Though a large body of information exists regarding the phytopathogenic genus *Colletotrichum*, ours is only the second reported entomopathogenic strain. It is not clear whether the colonization of an insect by this fungus is truly rare or a common but undetected event. Sexual recombination, observed *in planta* and *in vitro*, could be the means by which new genetic variants are generated leading to new biotypes with a selective advantage to colonize new hosts, which in this case is a novel host in a different kingdom.
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To my family with deep gratitude
“In wilderness is the preservation of the world” – Thoreau

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Sculpture by Antony Gormley, Sitka spruce forest, Scotland.
CHAPTER 1

Literature review

1. Introduction

The health of the eastern hemlock forests [Tsuga canadensis (L.) Carr.] in the northeastern US is in decline. Elongate hemlock scale (EHS), Fiorinia externa Ferris (Hemiptera: Coccoidea: Diaspididae) and hemlock woolly adelgid (HWA), Adelges tsugae Annand (Hemiptera: Adelgidae), have been identified as primary causes in the decline. Initial observations suggested that as A. tsugae invaded stands previously occupied by F. externa, the latter species could be displaced (McClure, 1997). However, the rapid spread of F. externa within the area of A. tsugae seems to contradict this assumption. Strong correlations have been found between A. tsugae and scale infestation levels (Danoff-Burg and Bird, 2002). F. externa can be more of a problem, even when relegated to a secondary role to A. tsugae since this species possesses a high fertility rate as well as an effortless mobility to new potential infestation spots by wind (transportation of crawlers up to 100 meters) and other mechanical agents. The unique shield-like cover or armor of this Diaspidid provides protection for the eggs and the adult from contact insecticides, natural enemies and adverse conditions. Because of its high reproductive rate, losses are soon replaced even with mortality rates of 95% (Baranyovits, 1953, Johnson and Lyon, 1988). The dense hemlock canopy makes complete coverage with pesticides difficult to achieve. This protection is enhanced because most of the scales occur in the lower part of hemlock needles. Systemic insecticides, such as Imidacropid, have also not been effective in controlling the pest (Hoover, 2003). Introduced predators
and parasitoids have not attained significant control of *F. externa* due to asynchrony between the natural enemies and pest. *F. externa* has become a destructive pest of hemlocks and as evidence of hemlock decline becomes more pronounced and widespread, public awareness and concern for the potential impact of this species has grown:

“While the hemlock woolly adelgid gets more of the publicity, other insects are contributing to the decline of the eastern hemlock. *Fiorinia externa* has probably caused more decline of hemlocks in terms of quick death than the *Adelges tsugae*”. (G. Hoover, pers. comm., 2004)

“Advanced stage of hemlock decline, as indicated by needle loss, was significantly correlated with *Fiorinia externa* abundance but not *Adelges tsugae* abundance, in eastern hemlock stands in the Black Rock Forest, Orange County, New York”. (Danoff-Burg and Bird, 2002)

“In recent years the populations of *F. externa* have increased dramatically and have contributed significantly to hemlock decline throughout much of the *A. tsugae* infested area. In the last 20 years *F. externa* has significantly expanded its geographical range”. (McClure, 2002a)

As hemlock declines in vigor, an open canopy allows an increase in understory light levels. As a result, hardwood species especially black birch (*Betula lenta* L.)
establish, leading to major landscape changes by transforming large areas dominated by conifers to young, rapidly growing hardwoods (Foster and O’Keefe, 2000). Moderate winter temperatures have favored the survival of *F. externa* and *A. tsugae* and populations of both species have increased. If dispersal of *F. externa* continues unimpeded and at the present rate, drastic changes in forest composition, landscape and ecological balance can be foreseen and ultimately the disappearance of the current hemlock stands and its historical, economical, environmental protective and recreational value could be lost.

A disquieting resemblance with chestnut stands (*Castanea dentata* (Marshals) Borkh.) in the early 1900s can be observed. The rapid spread of chestnut blight [*Cryphonectria parasitica* (Murrill) Barr] dictated the fate of the species and practically eradicated 3.5 billion chestnut trees from eastern North America in the following 40 years (Roane *et al.*, 1986; Anagnostakis, 1988; Bisiach, 1992).

### 1.1. Importance and decline of eastern hemlock

The eastern or Canada hemlock [*Tsuga canadensis* (L.) Carrière] is a native species of the eastern US and adjacent Canada. It is one of the more low light tolerant trees in New England forests (Burns, 1923; Anderson and Gordon, 1994) and one of the most long-lived in the eastern US, living up to 300 years (Godman and Lancaster, 1990; Foster, 2000). Eastern hemlock has been a dominant component of the New England forests for 8000 yr (Foster and Zebryk, 1993). The Pleistocene glaciations eliminated vegetation in the northeastern US and Canada, reducing hemlock to more moderate climatic conditions along the coast or in the foothills of the Appalachians (Anderson and Gordon, 1994). Pollen records show dramatic fluctuations of eastern hemlock in
northeastern forests suggesting that this species recovered after each fluctuation, regaining dominance in certain areas (Foster and Zebryk, 1993).

Eastern hemlock plays a key role in maintaining balanced, stable forest ecosystems. Their leaf litter produces a forest floor dominated by ammonium nitrogen, as opposed to the nitrate-based nitrogen of deciduous species, thus influencing long-term site quality and species dynamics (Anderson and Gordon, 1994; Mladenoff, 1995). They play a particular important ecological role along streams, where their shade provides shelter, sustains aquatic ecosystems and creates unique microclimate for forest wildlife (Howe and Mossman, 1995; Wydeven and Hay, 1995; Crow, 1995; Howard et al., 2000). The microclimate in pure eastern hemlock stands is substantially cooler than adjacent hardwood stands because of hemlock’s dense crowns. They grow in a wide variety of soil textures but most commonly in soils with impeded drainage (Tubbs, 1995). Hemlocks can persist longer than their more competitors and do not relinquish space easily.

Hemlock forests dominated by large trees on sheltered sites represent some of the oldest and more mature stands across southern New England (Foster and O’Keefe, 2000). There are ~930,000 ha of hemlock-dominated stands (*Tsuga* spp.) in North America. Associations may occur with other conifers and broadleaf tree species, such as the sugar maple (*Acer sacharum* Marsh.), American beech (*Fagus grandifolia* Ehrh.), yellow birch (*Betula allegheniensis* Britton), and American basswood (*Tilia americana* L.). They grow on a diverse range of sites but generally are associated with riparian environments. Hemlock’s natural range extends from northeast Minnesota across Wisconsin, northern Michigan, south-central Ontario to the extreme southern Quebec, and through New Brunswick and Nova Scotia. It is also found throughout New England, New York,
Eastern hemlock is second only to sugar maple in abundance within the northern forest, (Curtis, 1959). Old stands are divided between New England, with 54% of the hemlock area, the Mid-Atlantic states with 44%, and southern Appalachian states with 2% (McWilliams and Schmidt, 2000). The total net volume of hemlock species (Tsuga spp.) in the US is ~238 million cubic meters, representing 2% of the forest inventory within its natural range. About 96 million cubic meters of live hemlock species are found within the mixed maple-beech-birch forests. Around 60% of the harvested hemlock wood is used for pulp and paper production; the remaining is used for lumber (McWilliams and Schmidt, 2000).

A dramatic decrease in hemlock forests has been quantified and mapped from 1984 to 1994 using remote sensing techniques (Royle and Lathrop, 2002). Half of the range of hemlock along the eastern seaboard were infested with Adelges tsugae (Knauer et al., 2002). Tree mortality occurs five to six years after a heavy infestation (Mayer et al., 2002). Extended research on the impact of this pest on eastern hemlock has been reported (Bonneau et al., 1999; McClure and Cheah, 1999; Colbert et al., 2002; Orwig, 2002). The subsequent impact of hemlock mortality due to this pest in the ecosystem has been also documented (Orwig et al., 2002; Kimple and Schuster, 2002; Williams et al., 2002; Yorks, 2002). The most preoccupying characteristic related to the A. tsugae is its chronic nature. In contrast with other pest insects, A. tsugae persists, with no decline in the population after an outbreak (Souto and Shields, 2000).

The adelgid was first reported in the US in the Pacific Northwest in the 1920s. It apparently came from Japan where it is native, and was not a problem (Souto and
Shields, 2000). It was found in Virginia in the early 1950s and spread into New England in 1985. It is now widely established in practically all eastern coast states (Fig. 1.1), spreading at a rate of 20-30 km/yr, weakening and destroying hemlock stands in its wake (Orwig and Foster, 1998).

In spite of the obvious importance of *A. tsugae*, the elongate hemlock scale, *Fiorinia externa* (Homoptera: Coccoidea: Diaspididae), is also an obvious stressor factor responsible for the decline of hemlock which has been, so far, relegated to a secondary role as to the factors responsible for the current health situation of hemlock forest in the northeastern US.

The elongate hemlock scale is an armored scale. Armored scales are the most highly specialized plant parasites within the Coccoidea and usually attack perennial trees. They are also pests of fruit, nuts, shrubs and ornamental plants, including greenhouse and indoor plants. The worldwide production costs attributed to scales are estimated at 5 billion dollars annually, 500 million within the US (Kosztarab, 1996). Few countries in the world today are exempt of diaspids pests problems, which represent a serious menace to the citrus industry in most countries where this crop is cultivated (Jeppson and

**Figure 1.1.** Distribution of hemlock woolly adelgid, *Adelges tsugae*, in the eastern US (USDA Forest Service, 2004).
Carman, 1960). Their ability to successfully invade new territories has made them a threat in many parts of the world (Morrison and Renk, 1957; Morrison and Morrison, 1965; Russel et al., 1974; Kosztarab and Kosztarab, 1988). Extensive research covering all aspects of armored scales has been previously compiled (Helle, 1990 a, b).

*F. externa* is native to Japan (Takagi, 1963) and was accidentally introduced into the US in the 19th century. In Japan it is commonly found on *Tsuga diversifolia* Masters and *T. sieboldii* Carrière, only seldom causing damage (McClure, 1997). The *Fiorinia* scale was first collected in the US by F. N. Meyer on *Tsuga* sp. from Queens, Long Island, NY in 1908, and recorded as *F. japonica* Kuwana (Sasscer, 1912). It was later described by Ferris (1942) as a new species from material collected by H. S. McConnell in the area of Towson, north of Baltimore, MD, where the diaspidids were a local problem. The scale was also present in Ohio (1929), Connecticut (1929), Pennsylvania (1946), New Jersey (1950) and Washington, D.C. (1952) according to the United States National Museum records (Garrett and Langford, 1962b).

The greatest abundance of *F. externa* occurs within a 300 km radius of New York City (Danoff-Burg and Bird, 2002), spreading from southern New England to western Ohio and Tennessee (Fig. 1.2).

![Fig. 1.2. Distribution of elongate hemlock scale, *Fiorinia externa*, in the Eastern US (Johnson and Lyon, 1988; Hoover, 2003; Lambdin et al., 2005).](image-url)
The primary hosts are *T. canadensis*, *T. caroliniana*, *T. diversifolia*, *Abies* spp. and *Picea* spp., although it also feeds on *Cedrus* spp., *Pseudotsuga menziesii*, *Pinus* spp., and *Taxus* spp. (Hoover, 2003). The main host is eastern hemlock (McClure, 1988) but it can mature and reproduce on 57 different species among the native and exotic conifers (McClure and Fergione, 1977), although infested hemlocks are always near. Outbreaks of this pest often intensify following infestation of *A. tsugae*, drought or other stress factors. It may also co-exist with the circular scale [*Nuculapsis tsugae* (Marlatt)], another exotic species from Japan, however *F. externa* is a superior competitor due to the nutritional advantage gained by colonization of the tree earlier than its competitor hence monopolizing younger nitrogen-rich needles (McClure, 1980a; Reitz and Trumble, 2002).

1.2. The *Fiorinia externa* (Hemiptera: Coccoidea: Diaspididae) armored scale

Armored scales (Diaspididae) are characterized by a dorsal protective cover or shield, called the scale. It is composed of loose fibers secreted by pygidial glands, a fluid material discharged from the anus (thought to bind the fibers together) and two larval exuviae (one in the males), which are incorporated into the scale at each molt (Dickson, 1951; Disselkamp, 1954; Stoetzel, 1976). In the males the cover is a waxy fragile material and in the females it is hard and impermeable (Fig. 1.3).

In addition to the dorsal scale, most diaspids produce a membranous ventral scale composed of secretions from the ventral pygidial glands, plus incorporated ventral exuviae residuals (Beardsley and Gonzalez, 1975; Kosztarab and Kozár, 1988). The shape of the scale, its color and the position of the exuviae are relatively constant in a given species, and these characteristics are valuable for identification purposes (Gill,
In *F. externa*, the waxy covers can be observed on the lower surface of needles and on new cones (Johnson & Lyon, 1988; Hoover, 2003).

![Figure 1.3. F. externa adult female carrying eggs under translucent exuvia (A) and F. externa adult male with profuse filamentous strands (B).](image)

A distinct sexual dimorphism is characteristic in diaspids. The *F. externa* female is elongate, with margins almost parallel and tapering at the apex. The length of the adult varies depending upon the stage of development, from 1.4 mm in a newly-molted individual to 0.6 mm in a female that has completed oviposition (Wallner, 1965). The scale cover of the female is composed almost entirely of the sclerotized and elongate second exuvia (Ferris, 1942). The 2nd instar becomes heavily sclerotized, and the adult female within remains membranous. The female is found within the exuvia of the 2nd instar known as the pupillarial form (Sasscer, 1912; Davidson & McComb, 1958; Kosztarab, 1963). The adult uses the 2nd instar to protect the eggs in the same way that non-pupillarial forms use the scale covering (William and Watson, 1988). The female is more elongate than the male. The newly-molted adult female is pale yellow and gradually darkens from pale to dark brown whereas the male is whiter and narrower in shape (Duda, 1957; Wallner, 1965). The exuviae is translucent (Ferris, 1942). The old rostralis is left in the plant tissue. The scale cover of females is yellow-brown or tannish and is
2 mm long; in males it is slightly smaller (1 mm) and whiter than the females (Wallner, 1965). The covering will not drop off even after death, though it will erode with time. “The cover shows a specific peculiarity in that the first exuvia is exceedingly thin and transparent and appears to be detached from the second, to which it is connected only by a thin film of wax” (Ferris, 1942). Eggs (0.25 - 0.37 mm long) are laid under the 3rd instar exuvia. As egg laying progresses, the body of the female shrivels (Wallner, 1965).

Nymphs (crawlers) are flattened dorso-ventrally, 0.33 mm in length and are pale yellow-orange (Fig. 1.4). On the dorsal surface of the 2nd instar exuvia there are two long-necked glands that secrete wax (Wallner, 1965). Between the antennae (five segments, the distal being as long as the other combined), two other glands are present that secrete large amounts of filamentous wax, commonly called silk (Stoetzel, 1976). A glutinose substance is then secreted by the Malphigian tubules and discharged from the anus. This glues the filaments into a solid mass that hardens and forms an elongate cap over and around the insect’s body (Baranyovits, 1953; Stoetzel, 1976) which turns dark with age (Fig. 1.5). When these secretions are abundant, F. externa may be misdiagnosed as A. tsugae or spider mites (Hoover, 2003). Three pairs of legs are present. The rostralis is located between the first pair of legs (Wallner, 1965).

Crawlers are able to move a few meters, but once settled the 2nd instar starts feeding, becomes robust with a lemon yellow coloration and loses its legs. Females remain anchored for life (Baranyovits, 1953). The rostralis is composed of coiled stylets

Figure 1.4. F. externa crawler.
that are three or four times the length of the body (Johnson and Lyon, 1988). Males and females are not morphologically distinguishable up to the 2nd instar stage, which is the last feeding instar for males (Wallner, 1965).

“The third instar male scale is elongated (0.49 mm), white, with a terminal exuvia. The prepupal male is orange-yellow in color with 0.8 mm long. The antennae, wings and legs appear as everted sacs. The pupal male (0.85 mm) is dark orange in color and the appendages are hyaline. The fully developed male, prior to emergence, is contained under a scale cover composed of the first instar exuvia and a filamentous, white covering. It has formed new appendages: two pair of wings, the second one reduced to haltere-like structures, antennae and three pairs of legs and a conical head” (Wallner, 1965).

1.2.1. Life-cycle

*F. externa* overwinters as a fertilized female or in the egg stage. In early spring females start to deposit eggs beneath their waxy cover and may continue to lay eggs throughout the summer, only interrupted by cold weather (Johnson and Lyon, 1988; Hoover, 2003). One female can produce up to 20 eggs over the course of its life, though the average at any one time is six (Davidson and McComb, 1958; Stimmel, 1980).
The female contracts and occupies the anterior half of the last instar exuvia, providing space for the eggs (Wallner, 1965). Egg production is greatest from late April through May (Stimmel, 1980).

In 3-4 wk eggs hatch (early May) into nymphs (crawlers) that exit through an opening at the posterior end of the scale (Malinoski and Davidson, 2002; McClure, 2002b). They are very active and migrate preferentially to new needles (Davidson and McComb, 1958; Stimmel, 1980). Living crawlers have been observed at a distance of 105 m from infested trees (McClure, 1977b). Within 1-2 days after hatching, crawlers settle in the underside of a needle and insert their stylets in the needle, which penetrates between the epidermal cells or through a stomatal opening where the insect feeds on the mesophyll cells (Wallner, 1978; Hoover, 2003). The first instar nymph starts to secrete the waxy cover around it as it grows. Only the head region of the insect remains uncovered (Beardsley and Gonzalez, 1975).

Two types of waxy covering are produced: a frosty appearing layer from the posterior end which eventually covers the 1st and 2nd instars, and also a mass of trailing threads from two glands located between the antennae (Wallner, 1965). This latter secretion gives the tree the white-washed, mealy appearance. After 2-4 wk the insects’ body becomes flattened and the dorsal skin hardens and thickens to form the first scale cover (Wallner, 1965). They then molt into 2nd instars, and another 4 wk are required to reach maturity (Johnson and Lyon, 1988). Adults from this generation appear to reach maturity from mid to late July (Stimmel, 1980). The second stage male molts into a non-feeding prepupa and spins a cocoon, within which it pupates. When mature, males emerge as tiny winged insects, mate with the females and then die without feeding.
Mated females start to lay second-generation eggs (if present) six wk after mating (Hoover, 2003). Eggs produced later (June or July) hatch and mature in a shorter period of time (Stimmel, 1980).

*F. externa* completes two generations each year in the southern and Mid-Atlantic States, but usually only one in the Northeast (McClure, 2002b). The climate of the East has moderated in some areas, which may have allowed *F. externa* to become bivoltine in this region as well (McClure, 2002a). A summer and an incomplete autumn generation has been reported in Connecticut (McClure, 1977b, c) and New York (Wallner, 1964), two generations in Massachusetts (Bray, 1958) and Connecticut (Talerico *et al.*, 1967) have been reported and multiple generations in Maryland have been also documented (Davidson and McComb, 1958; Garret and Langford, 1969a). Overlapping stages can indicate either unsynchronized single generations or multiple overlapping generations (Stimmel, 1980). When a second generation occurs, individuals that develop from the eggs mature and a second outbreak of crawler occurs in September (Malinoski and Davidson, 2002). This new generation overwinters as fertilized females. Adult females may live up to one year (Johnson and Lyon, 1988).

1.2.2. Damage

Leaf chlorosis and other localized toxic effects are commonly associated with armored scale infestations (Beardsley and Gonzalez, 1975). *F. externa* feeds on the needles of their hosts by removing fluids from the mesophyll cells through piercing-sucking mouthparts (Wallner, 1965). The younger needles of the lower crown are highly preferred as feeding sites, but the scales can be found on needles of every age and throughout the crown (McClure and Fergione, 1977). When populations are high, *F.*
externa causes a chlorotic appearance of the upper needle surface, which is related to the destruction of mesophyll cells. Premature needle drop follows. In an advanced stage of infestation there will be thinning foliage. Dieback of major limbs, progressing from the bottom of the tree upwards usually occurs after F. externa densities reach 10 insects per needle (McClure, 2002b). The excessive loss of plant fluids will debilitate the tree and favor other pests such as the hemlock borer (Melanophila fulvoguttata Harris) or root rot (Armillaria spp.) and are readily broken or thrown by wind (Hoover, 2003). Death of the tree may occur in ~10 yr if populations are not controlled (McClure, 1980a).

1.2.3. Management

The management of accidentally introduced species is a difficult task. They frequently represent the most serious insect pests as they lack natural enemies capable of regulating their populations and maintaining them below an economic threshold. They rapidly spread throughout a defenseless host. The usual approach to the problem is to use insecticides or introduced natural enemies from the native geographic origin of the pest. Both of these options have limitations, challenges and potential negative environmental implications. Alternatives, such as extensive logging to remove infested trees, are impractical in many hemlock stands, as access is difficult and not cost effective. Systemic insecticides, such as Merit® (Imidacloprid), which are used to treat individual trees to manage A. tsugae, are not effective in managing armored scale insects (Hoover, 2003).

Tree health is directly correlated to the potential impact of a pest population, particularly in piercing and sucking insects such as F. externa. Hemlocks have shallow roots and thus are susceptible to drought stress (Fowells, 1965). Hence, hemlocks located within an infested area should be maintained in good health to discourage pest problems.
Applications of nitrogen fertilizer and broad-spectrum insecticides can aggravate the pest problem. Nitrogen enhances the survival, development rate and fecundity of scales, resulting in higher densities on fertilized trees (McClure, 1980b). Residues from insecticidal and fungicidal sprays and dusts in citrus foliage have been correlated with an increase of purple scale *Lepidosaphes beckii* (Newm.), and the Florida red scale, *Chrysomphalus aonidum* (L.) (Osburn and Spencer, 1938). Inert residues on leaves and fruit enabled the young crawlers to become established by protecting them from direct sunlight and heat, affording them a foothold among the residual particles.

In addition, an inadequate application of pesticides can cause resurgence of scale populations by eliminating natural enemies. The elimination of the primary parasitoid of the scale and three species of common predators from the lower crowns of partially treated trees after insecticide treatment has been recorded (McClure, 1977a). Attempts to find a practical solution for the management of hemlock scale have been tested for some decades with variable results.

### 1.2.3.1. Chemical control

Excellent control of *F. externa* crawlers was obtained with a malathion-DDT mixture and malathion sprays, (Davidson and McComb, 1958; Bray, 1958; Garrett and Langford, 1969b). However, the number of applications needed throughout the intermittent emergence period of crawlers made this control measure impractical. Control for up to 3 yr was reported when Dimethoate was applied as a foliage drench to the crowns of infested hemlocks (Wallner, 1962, 1965; Garrett and Langford, 1969b; McClure, 1977d). However, even if effective for ornamental trees, there are many negative impacts associated using insecticides, and because hemlocks are commonly
found near bodies of water, insecticides are not suitable for many environmental conditions. Complete coverage of a mature tree is difficult even spraying from underneath to reach the scales (Stimmel, 1980). Rapid resurgence of the scale after insecticide application commonly occurs (McClure and Fergione, 1977). Evidence has shown a density-dependent feedback affecting the scale abundance by which dense scale populations significantly limit the success of individuals of the following generations and high mortality of crawlers following pesticide spraying allows a rapid resurgence of the scale population. Observations also confirmed that scales which survived the treatment had significantly higher fecundity than untreated controls, probably because of improved host quality followed by a reduced herbivore pressure (McClure and Fergione, 1977; McClure, 1978a, 1979a).

1.2.3.2. Natural enemies: parasites and predators

The most important natural enemy of the *Fiorinia* scale is the aphelinid parasitoid, *Encarsia citrina* Craw [=*Aspidiotiphagus citrinus* Howard] (Hymenoptera: Aphelinidae) (Fig. 1.6). High parasitism rates in the native area of the scale, Japan, have been observed, with mortality rates ranging from 90.2 - 94.2% (McClure, 1986). This parasitoid maintains the scale at innocuous densities in Japan, however, similar results do not occur in the northeastern US though the parasitoid has been established for several decades and parasitism rates have intensified as scale populations have increased (McClure, 1977c). Lack of synchrony between the life cycles of the parasitoid and the host is believed to be the reason for the failure (McClure, 1978b).
Moderate numbers of the native lady beetle, *Chilocorus stigma* Say, (Coleoptera: Coccinellidae), have been recorded in infested areas of *F. externa* (Wallner, 1965). The adults and larvae attacked all stages of the scale. However, predation was not insufficient to control the scale population (Wallner, 1965). Recently, *Cybocephalus nipponicus* (Coleoptera: Nitidulidae), a native species of China and Korea, reared by the New Jersey Dept. of Agric. to control the Euonymus scale [*Unaspis euonymi* (Comstock)], has been introduced in Pennsylvania to test its potential for the control of *F. externa* (Blumenthal, 2003). The movement of this predator from the Euonymus scale to feed in other scales, including the Fiorinia scale had been observed. Releases and evaluations in Pennsylvania hemlock forests indicate that the beetle has the potential to be effective, over time, in controlling *F. externa* (Blumenthal, 2003).

### 1.2.3.3. Entomopathogenic fungi

The usefulness of fungi attacking scale insects has been exploited and studied extensively (Gossard, 1903; Rolfs, 1908; Berger, 1919, 1921; Watson and Berger, 1937; Fawcet, 1948; McCoy, 1985). Observations describing the occurrence of entomopathogenic fungi on scales have been reported since the end of the 19th century from all continents (Table 1.1). In the 20th century, 37 parasitic and semi-parasitic fungi

![Figure 1.6. Parasitized scale with *Encarsia* sp. (A) and single *Encarsia* sp. parasitoid (B).](image)
were reported on scale insects (Fawcett, 1948). The presence of entomopathogenic fungi specifically isolated from *Fiorinia* spp. also has been observed since the beginning of the 20th century (Table 1.2).

In 2001, an entomopathogenic fungus attacking *F. externa* and *N. tsugae* was reported (October 2001) in the hemlock forest at the Mianus River Gorge Preserve in Bedford, NY where an epizootic within the scale population was observed (McClure, 2002). Large sclerotia masses were found concealing in many cases the body of adult scales (Fig. 1.7).

No pathogens parasitizing *F. externa* had been previously reported. A group of entomopathogenic, phytopathogenic and endophytic fungi associated with *F. externa*, were isolated at the Entomology Research Laboratory, ERL (Marcelino et al., submitted). However, one fungus was the most commonly retrieved pathogen in the 36 sites from New York, Pennsylvania, Connecticut and New Jersey states, where collections of infected *F. externa* adults were made. This fungus was successfully inoculated in *F. externa*. Subsequent recovered of the fungus from infected dead scales revealed a potential use of this fungus for biological control.

Colonies of this fungus present a remarkable morphological plasticity, growing in radial non-uniform patterns and producing different pigmentation ranging from whitish-pink to intense black. Black hemispherical sclerotia, approximately 2 mm wide have been observed in the field. SDYA 1/4 media (Sabouraud's dextrose yeast agar 1/4 strength), with low concentration of carbohydrates, causes formation of the same sclerotious bodies
found in the field, after four days of incubation. Conidial spores have an elliptical shape (6.51-6.73 × 3.26-3.35 µm) initially with round edges in young cultures and progressively acute as the culture reaches full growth (Gouli et al., 2004). The genus was preliminary identified by scientists at the Entomology Research Laboratory, UVM (S. Gouli and V. Gouli, pers. comm. 2003) as Hypocrella sp. and the species was verified as the anamorph Aschersonia marginata Ellis and Ever (Ascomycota: Hypocreales: Clavicipitaceae) by Dr. Zengzhi Li (Head of Entomogenous Fungi Branch of the Mycological Society of China). Doubts concerning the identification remained after contacts with worldwide renown taxonomists (Table 1.3 for identifications), since Aschersonia spp. mainly grows in tropical or sub-tropical climates (see Appendix 1 for genus description), very different from the northeastern US climate. Molecular analysis allowed an accurate identification of the genus as Colletotrichum.

The genus Colletotrichum Corda (Ascomycetes: Phyllacorales: Phyllachoraceae) described by Sturm (1831) comprises one of the most extensive fungal groups of phytopathogenic species in the world, being a direct cause of plant anthracnose and damping-off, blight and spot disease in infected plant tissues, fruits, stems, roots and flower petals (Latunde-Dada, 2001). Entomopathogenic isolates of Colletotrichum spp. have only been reported to occur on Orthezia praelonga Douglas (Hemiptera: Ortheziidae), a major scale pest of Citrus spp. in Brazil (Robbs et al., 1991). Colletotrichum gloeosporioides causes an intense epizootic in populations of this insect (Cesnik and Oliveira, 1993) confirming an early Brazilian report (Batista and Bezerra, 1965).
The ubiquitous presence of this unreported entomopathogenic strain of *Colletotrichum* in the epizootic areas led us to focus our attention and research in the biological processes involved in the interactions between the insect pest (*F. externa*) and fungal pathogen (*Colletotrichum* sp.) and to determine the origin and biocontrol potential of this entomopathogenic fungus.

1.3. **Entomopathogenic fungi as biological control agents**

Pathogens have long been known to play a major role in the population dynamics of many important forest pests. Among the causal agents of diseases in insects, such as protozoans, bacteria, viruses, rickettsia and nematodes, the entomogenous fungi (entomopathogenic and entomoparasitic fungi), obligate or facultative parasites of insects, play a relevant role. Because of their conspicuous macroscopic growth on the surface of their hosts, fungi were the first microorganisms found to cause disease in insects (Tanada and Kaya, 1993). Economically, the use of entomopathogenic fungi offers a unique alternative to a chemical control approach of an insect pest. Under ideal conditions an area of 1 ha can be treated with fungal formulations at a cost of $20 (Wraight *et al.*, 2001). In addition, nontarget safety testing increases the registration cost of a chemical pesticide to an estimated $20-40 million (Marrone and MacIntosh 1993), contrasting with the approximately $2 million for a biological control agent (Rodgers, 1993). Successful biocontrol has been extensively reported (Campbell *et al.*, 1985; Fransen, 1987; Lewis *et al.*, 1996; Lacey *et al.*, 1996; Charnley, 1997; Keller *et al.*, 1997; Inglis *et al.*, 2001; Meekes *et al.*, 2002) as well as commercial myco-insecticide use (see product list in Baron, 2002; IR-4, 2005). The most outstanding achieved control, up to
now, was the introduction and establishment of the entomopathogen *Entomophaga maimaiga* Humber, Shimazu & Soper, which has caused a massive epidemic in northeastern US gypsy moth (Lepidoptera: Lymantriidae) populations. This pest has been maintained below the economical threshold for several years due largely to this fungus (Hajek *et al*., 1996; Dwyer *et al*., 1998). There are at present 74,000 species of fungi identified worldwide (Hawksworth, 2001), a portion of which are insect-specific (Rossman, 1994). The number of fungi associated with insects is so vast and their role so relevant, that their inclusion in biodiversity projects is of primary importance (Benjamin *et al*., 2004). Exploitation of pathogenic organisms, under suitable environmental conditions, may be an important tool to maintain an insect’s population under an economic threshold. If the parasitic pathogen subsequently establishes and successfully maintains its viability in the environment, it can become a practical pest management option.

Records of entomopathogenic fungi from the Oligocene-Miocene boundary (~ 25 million yr old) have been reported, indicating fungi pre-date human existence (Poinar and Thomas 1982, 1984). In the late 19th century, research directed towards the taxonomy of entomopathogenic fungi increased. Petch (1870-1948) in Asia, and Mains (1890-1968) in America, led the field of fungal taxonomy (Smith, 1969). Intensive research focusing on the use of fungi for the control of agricultural pests began in the 20th century (Samson *et al*., 1988). This period was followed by a decline phase in the mid-1950s. Synthetic organic pesticides, epitomized by DDT, became the dominant control technique after 1945, especially for the control of forest insect pests (Perkins, 1988). Synthetic insecticides have remained the primary control method but increased attention directed
towards biological control methods began in the 1980s (Ignoffo and Anderson, 1979; Burges, 1981; Hall and Papierok, 1982; Jaques, 1983; Rombach and Gillespie, 1988), stimulated by the general awareness of insect resistance to chemical pesticides and the environmental hazards of chemical applications. The potential dangers of pesticides were reported by governmental agencies, including the National Research Council of the National Academy of Sciences, and by various congressional committees (Knipling, 1979). Many popular books on the subject were written, including Rachel Carson’s ‘Silent Spring’ (1987).

Examples of successful biological control with bacteria and fungi have been reported in North America (Burges and Hall, 1982) and England (Wilding, 1983). At present, the commercialization of mycoinsecticides is no longer a pioneering field but a common resource. The forestry market was one of the first in which a microbial agent (Bacillus thurigiensis subsp. kurstaki) replaced broad-spectrum chemical insecticides (Frankenhuyzen et al., 2000). Sales of the bacterium Bacillus thuringiensis for insect control represented over 90% of the international biocontrol market in the 1990s (Rodgers, 1993).

Of the ~700 species of entomopathogenic fungi distributed in 100 genera, a few are in use or under development for insect control (Lisansky and Hall, 1983; Roberts and Wraight, 1986, McCoy et al., 1988) in agriculture, forestry or medical fields. Genera containing entomopathogenic species were compiled in the ‘Atlas of Entomopathogenic Fungi’ (Samson et al., 1988) and by McCoy et al. (1988). Later Humber (1989, 1992, 1997 and 1998) and Mueller et al. (2004) compiled the most characteristic conditions in
which a fungus might occur to cause infection in insects, which is spore-bearing states (Fig. 1.8).

Adapted from:

Figure 1.8. Taxonomic classification of entomopathogenic fungi

The phylum Deuteromycota contains the largest number of entomopathogenic fungi, including genera with a wide geographic distribution (Beauveria spp., Metarhizium spp.), comprising primarily mycopathogens of soil insects, i.e. Beauveria bassiana
Species with a main tropical or subtropical distribution (*Gibellula* spp. and *Hymenostilbe* spp.) and genera presenting host group specificity, such as *Nomuraea rileyi* (F.) Samson to Lepidoptera, or *Aschersonia* sp. to scales and whiteflies are also present in the Deuteromycota, (Zimmermann, 1986; Humber, 2000). The Basidiomycota (*Septobasidium* sp. Pat.) are absent from the list since evidence indicates they essentially live symbiotically with the insect (Samson *et al*., 1988). *Septobasidium* sp. constitutes a distinct evolutionary shift from the common entomopathogenic pattern of activity of fungi where death overcomes quickly after host infection (Humber, 1984). However, pathogenic representatives of *Septobasidium* sp. are known (Evans, 1989).

For a thorough understanding of the principles of insect pathology, a detailed knowledge of the structure and biology of the infective agent is imperative. Unlike other insect pathogens, entomogenous fungi initiate infection by attachment and germination of the spore which is able to penetrate the cuticle of the host insect. Infection from ingested spores is a rare occurrence (Roberts and Humber, 1984). Entomopathogenic fungi have spores adapted to attach to an insect host, but the physical and chemical characteristics of spore and cuticular surfaces responsible for attachment are unknown (Roberts and Humber, 1981). The adhesion of the fungal spore to the insect cuticle is a critical phase in the infection process. Hypovirulence can result from a deficient attachment of spores. Many aspects are involved in the adhesion process such as the physiological state of the host, chemical nature of the cuticle surface, microclimatic conditions of host cuticle and host specificity (Boucias and Pendland, 1983; Farges, 1984).
After adhesion, the penetration of the insects’ cuticle (involving both toxin and enzyme production) occurs, either directly by germ tubes or by infection pegs which evaginate to form appressoria. After penetration into the insect’s body, a multiplication phase (hyphal bodies) takes place in the haemocoel (Roberts, 1981). After filling the host with mycelium, hyphae grow in the insect’s integument. Finally, production and dissemination of spores initiates on the external surface of the host (Poinar and Thomas, 1978). After death, the insect’s body becomes hard and mummified, containing the fungal stroma. Dead insects are generally attached to the subtract where they were infected (e.g. plants) by their appendages or proboscis, or by rhizoids or pseudocystidia produced by the fungus (Tana and Kaya, 1993). When an infection occurs naturally, without human intervention, such as the present case in study, and the entomopathogenic fungi rises to an epizootic level, control can be impressive. Unfortunately, this situation usually occurs after the economic threshold has been reached.

The current epizootic occurring naturally within population of *F. externa* in the northeastern hemlock forest of New York, Pennsylvania, Connecticut and New Jersey states, is an exceptional opportunity to study the etiology of the most commonly isolated microorganisms from infected scales within the geographic distribution of the epizootic, *Colletotrichum* sp.

1.4. The genus *Colletotrichum*

*Colletotrichum* spp. are a direct cause of anthracnose in a vast number of plant species (Latunde-Dada, 2001). The common symptoms of disease are dark, sunken, lenticular necrotic lesions that occur in developing and mature tissues, usually containing
the acervuli of the fungus (Prusky et al., 2000; Latunde-Dada, 2001). *Colletotrichum* spp. can also be saprophytic or secondary invaders of moribund tissue (Waller, 1992; Mills, 1994).

This genus is widely used as a model system for molecular and phytopathogenesis studies comprising infection processes, resistance mechanisms, mycoherbicide use, quiescence, biochemistry and molecular biology of host specificity (Prusky et al., 2000; Rodriguez and Redman, 2000; Latunde-Dada, 2001). *Colletotrichum* and *Phytophthora* are the most studied plant pathogenic fungi in the world (Prusky et al., 2000). The revision by von Arx (1957) was a relevant milestone in the taxonomy of the genus, reducing the existent 750 species to 11 taxa based on morphological characteristics. The use of DNA techniques allowed a more accurate examination of the different species and taxa considered by von Arx (1957). Host specialized forms were identified as distinct species by Sutton (1992) who reported a total of 39 species in this genus. At present there is a lack of consensus in the scientific community regarding the number of species in the genus *Colletotrichum* (Cannon et al., 2000). It is currently undergoing intensive revision and clarification with the use of molecular taxonomic techniques. Species complexes and subspecific groups recently have been proposed (Latunde-Dada, 2001).

1.4.1. Taxonomic considerations

Genetic and mating type studies indicate that the population structure and dynamics of *Colletotrichum* spp. is complex (Chacko et al., 1989, 1990; Cisar et al., 1994, 1996; Roca et al., 2004a, 2004b). Sexual compatibility and outcrossing between isolates of the same species and between species with different host specificities are the likely cause of the high degree of variability observed (Baxter et al., 1985; Cisar et al.,
1994). Genotypic diversity is enhanced mainly by outcrossing rather than inbreeding (Frank, 1992).

Morphologically-similar races of a *Colletotrichum* sp. pathogen are characterized in the basis of physiological parameters such as adaptation to different hosts (see Ansari *et al.*, 2004). They are then separated into biotypes called *formae speciales* (Shoemaker, 1981; Cisar *et al.*, 1994). These biotypes are considered to be genetically distinct, although evidence of chromosome transfer suggests that genetic exchange between biotypes can occur in the field (Masel, 1996). Several *Colletotrichum* spp. may be associated with a single host and a single *Colletotrichum* sp. may infect multiple hosts (Freeman *et al.*, 1998). There is uncertainty whether *Colletotrichum* isolates from one host can successfully colonize and infect another host or if they are host-specific (Vaillancour and Hanau, 1992).

For example, *Colletotrichum gloeosporioides* f. sp. *aeschynomene* has been tested for pathogenicity in 30 different plant species and 46 cultivars of economic relevance, and also in wild plants in the Leguminosae, in order to assess the specificity, biological control potential and commercialization potential of this strain against *Aeschynomene virginica* (Templeton, 1984). The only susceptible species were the target weed *A. virginica*, and a related weed, *Aeschynomene indica* (Daniel *et al.*, 1973). The pathogen was released commercially in 1982 as Collego™ (Weidemann, 1991). However, TeBeest (1988) tested this same *Colletotrichum* species in 77 plants comprising 43 genera in 10 families, reporting that five genera in the subfamily Papilionidae included susceptible species to the fungus, although susceptibility diverge, with only the target weed *A.*
virginica being killed. The relationship between plant species and the pathogen specificity is unclear (Weidemann, 1991).

The complete host range of most *Colletotrichum* species is largely unknown (Fagbola and Abang, 2004). Pathogens with a wide range of hosts, such as *C. gloeosporioides*, can have special forms (*formae speciales*) with more limited host ranges (Weidemann, 1991). Three major species aggregates of *Colletotrichum* are currently recognized: *C. gloeosporioides*, *C. acutatum* and *C. orbiculare*. Further reassembling will probably confirm two more groups, *C. capsici* and *C. graminicola* (Cannon *et al.*, 2000).

The concept of species in the genus *Colletotrichum* is currently being debated (Sutton, 1992). Morphological characteristics initially used to determine species within the genus (i.e. shape and size of conidia, setae, and appressoria) are widely recognized as inadequate (Sherriff *et al.*, 1994; Sreenivasaprasad *et al.*, 1996; Cannon *et al.*, 2000). For example, *C. acutatum* was considered a morphological variant and synonymous with *C. gloeosporioides* until the Simmonds revision of the genus in 1965 (Cannon *et al.*, 2000). In 1957 the genus was again revised and >600 synonyms of *C. gloeosporioides* were found (von Arx, 1957). Many contained morphological and physiological differences. The von Arx report was then revised and *C. gloeosporioides* was described comprising seven *formae speciales* (Sutton, 1992). Wide morphological plasticity in culture including pigmentation differences, growth rate and overlapping phenotypes have been consistently reported (Baxter *et al.*, 1983; Walker *et al.*, 1991; Freeman and Rodriguez, 1995; Lardner *et al.*, 1999; Prusky *et al.*, 2000; Sanders and Korsten, 2003; Couto and Menezes, 2004). Recently, vegetative compatibility groups (González and Sutton, 2004) and molecular analyses have been used to determine diversity within species (Freeman *et al.*
Evolutionary relationships between *Colletotrichum* lineages also have been studied, confirming the genetic diversity within this genus and the difficulties to clade species based on genetic parameters (Crouch, 2006).

1.4.2. rDNA analyses to discern *Colletotrichum* spp.

In the last decade, development of molecular techniques, coupled with traditional methodologies have been used to clarify the phylogenetic relationship within the genus *Colletotrichum* (Mills *et al*., 1992; Johnston and Jones, 1997; Balardin *et al*., 1999; Freeman *et al*., 2001a; Afanador-Kafuri *et al*., 2002; Ureña-Padilla *et al*., 2002; Talhinhas *et al*., 2002). As an example, sequence analysis of rDNA has enabled the identification of multiple genotypes of *C. acutatum* (Förster and Adaskaveg, 1998; Freeman *et al*., 1998) and also clonal sub-populations differing in colony appearance (pink vs. gray cultures), conidia morphology, virulence, temperature relationship for growth and genotype (Adaskaveg and Hartin, 1997; Förster and Adaskaveg, 1998). Population diversity within this species in New Zealand from fruit rots, lupin and pine, led to the determination of *C. acutatum* as a species group (*Colletotrichum acutatum sensu lato*). Host specificity (Lardner *et al*., 1999) and molecular analyses made it possible to distinguish four *C. acutatum sensu stricto* sub-groups (Johnston and Jones, 1997). Subsequently two additional distinct groups have been found (Guerber *et al*., 2003). A comprehensive molecular analysis of *C. acutatum sensu Simmonds* (one of the sub-groups within *C. acutatum sensu stricto*) has been conducted (Freeman *et al*., 2001). The phylogeny and systematics of 18 *Colletotrichum* spp. led to the conclusion that *C. acutatum* shows the maximum intraspecific divergence in the ribosomal DNA internal transcribed spacer.
(ITS) and *C. capsici* the greatest interspecific divergence (Sreenivasaprasad *et al.*, 1996). The cosmopolitan host range of this species is repeatedly assessed and its host range expanded (Sreenivasaprasad and Talhinhas, 2005).

Analysis using Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP) of the 28S, 18S, 5.8S ribosomal subunits (coding and conserved) and ITS regions (non-coding and variable) have also been conducted on fungal species identification processes (Bailey *et al.*, 1996; Martinez-Culebras *et al.*, 2003; Fagbola and Abang, 2004). The ribosomal internal transcribed spacers I and II (ITS1 and ITS2) have been widely used as a base for *Colletotrichum* species differentiation (Freeman and Rodriguez, 1995; Freeman *et al.*, 2000; Moriwaki *et al.*, 2002; Martinez-Culebras *et al.*, 2003). The 18S and 28S regions are functional and more conserved, although some parts (especially D1 and D2 domains) are known to be variable (Cannon *et al.*, 2000). Conserved gene regions can be used for phylogenetic analysis at the genus level and above (Mindell and Honeycutt, 1990; Hills and Dixon, 1991).

The ITS regions play a role in forming the three-dimensional structure of ribosomes, because these regions do not interact and display more variation. Thus, they are valuable tools for differentiation of taxa at and below the species level (Cannon *et al.*, 2000). In *Colletotrichum* spp. the ITS1 region, with 50.3% variable sites, has a greater intra- and inter-specific divergence than the ITS2 region with 12.4% variable sites (Sreenivasaprasad *et al.*, 1996). For differentiation within *Colletotrichum* spp. relatively low variation in ITS sequences may occlude intraspecies diversity. Therefore, the introns of the glutamine synthetase (GS) and the glyceraldehydes-3-phosphate dehydrogenase
(GPDH) genes have been used to identify genetic differences (Guerber et al., 2003). These DNA regions are valuable phylogenetic tools to identify inter-and intra-specific diversity in Colletotrichum spp. (Liu and Correll 2000; Liu et al., 2001) and for marker gene expression and transformation studies with the teleomorph of Colletotrichum, Glomerella (Templeton, 1992a). Molecular data are used to create phylogenetic trees that approximate to the evolutionary history (Berbee and Taylor, 2001).

1.4.3. Hosts

1.4.3.1. Plants

The range of hosts within the genus Colletotrichum is wide, and reports of new host plants are common. The genus shows a wider expression in warm moist environments such as the humid and sub-humid tropical zones (Mills et al., 1992; Waller, 1992). Forty seven cultivated host species in South Africa have been reported to be hosts of Colletotrichum spp. (Gorter, 1977 cit. in Baxter et al., 1983). In New Zealand 39 host species from 23 plant families also have been reported (Simmonds, 1965). Xiao et al. (2004) recovered Colletotrichum spp. from 23 cultivated and non-cultivated host plant species in west-central Florida, US. In Japan, Moriwaki et al., (2002) isolated 25 Colletotrichum species from 123 plant species including Gramineae, Leguminosae and Cucurbitaceae. An extensive record of hosts (grain legumes, pasture legumes, trees) was made by Lenné (1992), assessing at least nine species of the fungus within 102 plant species.

Economically significant losses occur in cereals, grasses, legumes, woody plants, trees, vegetables and perennial crops worldwide as a result of Colletotrichum spp. (Lardner et al., 1999). The complex of Colletotrichum spp. causing anthracnose disease is
the most serious threat to the strawberry industry (*Fragaria x ananassa* Duchesne). *Colletotrichum fragariae* Brooks was first reported on strawberry in the US by Brooks (1931). Later, *C. acutatum* Simmonds *ex* Simmonds (Simmonds, 1965; Smith and Black, 1986) and *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (teleomorph *Glomerella cingulata* [Stonem.] Spauld. & H. Schrenk.) were reported to cause anthracnose disease in strawberry, damaging roots, stolons, seeds, petioles, immature leaves, buds, fruits, flowers and causing crown rot in all cultivated strawberries worldwide (Kulshrestha et al., 1976; Gunnell and Gubler, 1992; Howard et al., 1992; Freeman and Katan, 1997; Tanaka and Passos, 1998; Tanaka et al., 2001; Mertely and Legard, 2004; Horowitz et al., 2004). Some of these diseases do not produce the dark sunken lesions characteristic of anthracnose and a more general description of *Colletotrichum* strawberry diseases, is proposed (Legard, 2000). *Colletotrichum acutatum* is a quarantine organism on strawberry in the European Union (Parikka and Lemmetty, 2004). It has been a quarantined organism in Canada until 1997 when this measure was deregulated (Canadian Food Inspection Agency, 1997). The morphology and cultural characteristics, infection and pathogenicity mechanisms of *Colletotrichum* spp. on strawberry have been documented (Smith, 1990; Horowitz et al., 2004).

*Colletotrichum gloeosporioides* has been reported to cause losses of 35-70% in sweet and hot peppers (Alexander and Marvel, 2001). *Colletotrichum sublineolum* causes economical loses in sorghum (Khan and Hsiang, 2003) and *Colletotrichum graminicola* (Cesati) Wilson causes anthracnose disease on several economically important crops including maize (Nicholson, 1992; Bergstrom and Nicholson, 2000), annual blue grass (*Poa annua*) and bent grass turfs (*Agrotis* spp.) on golf courses in the northern US,
Canada and Europe (Browning et al., 1999) and other turfgrasses (Khan and Hsiang, 2003).

*Colletotrichum coccodes* (Wallr.) S. J. Huges is an important pathogen in potato production causing anthracnose on tuber surfaces (Tsror et al., 1999; Lees and Hilton, 2003). It is also important in tomato production causing anthracnose in fruits and roots (Byrne et al., 1997; Dillard and Cobb, 1998). Fifteen species of weeds growing between crop rotations have been found to serve as hosts for *C. coccodes* (Raid and Pennypacker, 1987).

A common characteristic of *C. sublineolum, C. graminicola* and *C. coccodes* is the development of black micro-sclerotia within infected tissue (Byrne et al., 1997). *Colletotrichum truncatum* (Khan and Sinclair, 1992), *C. coccodes* (Sanogo and Pennypacker, 1997), *C. acutatum* and *C. gloeosporioides* (Dingley and Gilmour, 1977) produce sclerotia in culture. The latter two species produce, respectively, poorly developed sclerotia and abundant sclerotia.

Proteaceae plants, valuable commercially and sought after as cut flowers, are also hosts of *Colletotrichum* spp., causing seedling damping off, anthracnose, leaf and stem lesions (Lubbe et al., 2004). *Colletotrichum trifolii* is an important pathogen for alfalfa legume crops (Dickman et al., 1995). *Colletotrichum truncatum* causes yield losses of lentil in the northern US (Venette et al., 1994) and Canada (Ford et al., 2004). Lupin (*Lupinus* spp.) crops valuable for their high seed protein content are also affected by anthracnose (Nirenberg et al., 2001; Talhinhas et al., 2002). *Colletotrichum lindemunthianum* (Sacc. & Magnus) Briosi & Cav., is a serious disease in beans (*Phaseolus vulgaris* L.) with losses of 95% in susceptible cultivars (Guzman, 1979; Tu,
Colletotrichum demantium is the causal agent of anthracnose in cowpea stems (Smith and Aveling, 1997).

Perennial crops are highly susceptible to Colletotrichum species. The most consistently reported hosts are the following: avocado (Freeman et al., 1996; Everett, 2003; Sanders and Korsten, 2003), banana (Couto and Menezes, 2004), cashew (Muniz et al., 1998), cocoa (Dodd et al., 1992), coffee (Sreenivasaprasad et al., 1993; Nachet and Abreu, 2002), cotton (Bailey, 1996), kenaf and jute (Waller, 1992), mango (Afanador-Kafuri et al., 2002; Sanders and Korsten, 2003), papaya (Waller, 1992), passiflora (Afanador-Kafuri et al., 2002), pepper (Ivey et al., 2004), rubber (Fernando et al., 2000), sugarcane (Wallner, 1992), tamarillo (Afanador-Kafuri et al., 2002), tea (Waller, 1992) and tobacco (Waller, 1992). Colletotrichum gloeosporioides and C. acutatum are the most important pathogens. Fruit and nut producing trees with anthracnose caused by the fungus have been also reported: almond (Förster and Adaskaveg, 1999; Freeman et al., 2000), peach (Adaskaveg and Hartin, 1997; Ureña-Padilla et al., 2002) and citrus (Zulfiqar et al., 1996; Timmer and Brown, 2000; Moretto et al., 2001). Fruit-rots caused by Colletotrichum spp. were observed in apple, fig and pear (Johnston and Jones, 1997), olive (Martin and García-Figueres, 1999) and grapes (Talhinhas, 2002). Deciduous trees have been reported hosts. Canker and dieback of grafted Japanese maple (Acer palmatum) was reported in a nursery in Connecticut, US and the cause was an infection by C. acutatum (Smith, 1991b; Smith, 1993).

Conifer trees also have been reported as hosts. Colletotrichum acutatum was reported affecting four month old seedlings of western hemlock [Tsuga heterophylla (Raf.) Sarg.], western red cedar (Thuja plicata Donn), and Sitka spruce [Picea sitchensis
(Bong.) Carr.] in British Columbia, Canada (Hopkins et al., 1985). A subsequent study in nurseries where infections were observed showed the disease was confined to 380 western hemlock seedlings (Hopkins et al., 1985). *Colletotrichum acutatum* f. sp. *pinea* was found to cause temporary stunting from which seedlings normally recovered within the first year (Canadian Food Inspection Agency, 1997). Surveys in outplanted sites with western hemlock seedlings on Vancouver Island revealed the presence of *C. acutatum* in 70,000 seedlings (Hopkins et al., 1985). *Colletotrichum gloeosporioides* was also reported to cause blight in ~250,000 greenhouse grown western hemlock seedlings (70 d old) near Vancouver, Canada (Lock et al., 1984). Disease susceptibility to *C. acutatum* was assessed in western hemlock and mountain hemlock, being 95% and 92%, respectively (Griffin et al., 1987). Seedlings of *Pinus radiata* D. Don were found infected by *C. acutatum* f. sp. *pinea* Dingley & Gilmour causing terminal crook disease in New Zealand (Dingley and Gilmour, 1977; Nair and Corbin, 1981; Nair et al., 1983). Although initially it was suggested a spread of the pathogen from the co-cultivated tree lupin, *Lupinus arboreus* (used to provide nitrogen for young *P. radiata* plantations), pathogenicity tests assessed the host specificity, finding isolates collected from lupin were different from those collected from pine (Lardner et al., 1997).

### 1.4.3.2. Mammals and amphibians

Although rare, mycosis caused by *C. gloeosporioides* has been reported in humans, mainly opportunistic infection in previously open injuries (Guarro et al., 1998, Cano et al., 2004). Keratitis has been reported to be caused by *C. graminicola* in humans, usually after eye injury (Ritterband et al., 1997) and a mycosis by *C. acutatum* in amphibians also has been observed (Manire et al., 2002).
1.4.3.3. Insects

Biological control of *O. praelonga* (Hemiptera: Coccoidea: Ortheziidae) with the fungus *C. gloeosporioides* f. sp. *ortheziidae* has been used for the last 13 years in Brazil (Robbs *et al*., 1990, 1991, 1993; Viegas *et al*., 1995; Cesnik and Ferraz, 2000). Mortality rates in this scale insect, following applications of suspensions of *C. gloeosporioides* f. sp. *ortheziidae*, ranged from 42-82% after 35 d and 85-96% 70 d post-application (Cesnik *et al*., 1996). Assays were developed to evaluate the effect of the isolates on *Citrus* spp. They were not pathogenic to field-grown *Citrus* spp. plants but were to these grown in greenhouses, although the fungus was commonly recovered from treated plants in the field (Teixeira *et al*., 2001). Peach, apple and pumpkin leaves were also evaluated and determined to be susceptible to all isolates if they were previously injured (Teixeira *et al*., 2004). No toxicity to Crustaceae (Jonsson and Genthner, 1997) or mice (Castro *et al*., 1998) was found.

Scientists at Embrapa Meio Ambiente, Jaguariúna, Brazil, studied the control of *O. praelonga* from 1992 to 2003 and reported they have found no other fungus with the same infectivity to *O. praelonga* as *Colletotrichum* during their intensive field testing (Cesnik *et al*., 2005). The large scale production of the fungus has been limited by bureaucratic constrains due to the reports of phytopathogenic strains of this fungus (Cesnik *et al*., 2005), however commercialization in underway (R. Cesnik, pers. comm.). Studies on the biochemistry and phytopatogenicity of the entomopathogenic isolates from *Orthezia* have shown a distinct divergence from the known phytopathogenic isolates (Rosamiglia and Melo, 1996).
Benoit *et al.* (2004) recently reported isolation of *C. acutatum* from internal mycoflora of the cave cricket, *Hadenoecus cumberlandicus* (Hemiptera: Rhaphidophoridae), suggesting the pathogen might have been acquired during plant foraging. However, re-evaluation of the fungus showed no effect on insect survival (Benoit, pers. comm.).

### 1.4.4. Biocontrol of weeds with *Colletotrichum* spp.

Weeds cause major problems in agriculture systems worldwide, reducing nutrient intake for vigorous crop growth and thus reducing the quality and market value in crops (TeBeest *et al.*, 1992). Five strains of *Colletotrichum* spp. have shown commercial potential and 19 strains have been researched as possible mycoherbicides (Morris, 1983; Mortensen, 1988; Charudattan, 1991; Makowski and Mortensen, 1992; Templeton, 1992b). Biocontrol programs using *Colletotrichum* spp. as a mycoherbicide are being developed in North America, Europe and Asia.

Collego™ (Encore Technologies, US), a dry powder formulation with *C. gloeosporioides* f. sp. *Aeschynomene* has been commercially available since 1982 for control of *Aeschynomene virginica* (L.). This weed is responsible for crop losses in rice (*Oryze sativa* L.) and soybeans (*Glycine max* L. Merr.) in Arkansas, Louisiana and Missouri, US (Bowers, 1986; Templeton, 1992b). When compared to chemical herbicides, Collego™ was nearly as effective (Yang and TeBeest, 1993). From 1982 to 1988 farmers treated 8000 ha/yr of rice and soybeans (Smith, 1991a). Production was stopped in 2001 due to market constrains (D. Goulet, pers. comm.). Collego™ (active ingredient, *Colletotrichum gloeosporioides* f. sp. *Aeschynomene*) and DeVine™ (active ingredient, *Phytophthora palmivora* (Butler) Butler] (Charudattan, 1991) were the first
fungal herbicides introduced for commercial use in the US. Until 1996, only BioMal® was registered for commercial use, applying another strain of *Colletotrichum* spp. as the active ingredient (Hoagland, 1996).

BioMal® (Philom Bios Inc.) was registered in Canada in 1992, but has not been commercially used (G. Hnatowich, pers. comm.). It has been tested for the control of Malvaceae weeds in flax and lentils. The active ingredient of the dry formulation is *C. gloeosporioides* f. sp. *malvae* (Makowski and Mortensen, 1992; Morin *et al*., 1996). The management of *Xanthium* weeds, noxious to pastures and crops, with *Colletotrichum* spp. has been investigated (McRae and Auld, 1988; Walker *et al*., 1991). Burr Anthracnose® using *C. orbiculare* as the active ingredient was tested for control of spiny cockburr (*Xanthium spinosum* L.) in Australia, but it is also not commercially available (Templeton, 1992b).

Velvetleaf (*Abutilon theophrasti* Medik) is a major annual noxious weed in soybeans and corn (*Zea mays* L.) in the US, Canada and Europe (Warwick and Black, 1988). Strains of *Colletotrichum coccodes* selectively attack these crops (Wymore *et al*., 1988). *C. coccodes* was initially studied as an application combined with chemical herbicides. The mixture increased the spore viability of the fungus (Wymore and Watson, 1986; Wymore *et al*., 1987). Subsequent research focused on the effect of *C. coccodes* in the intra-and inter-specific competition potential between velvetleaf and soybean plants (DiTommaso and Watson, 1995; DiTommaso *et al*., 1996). *Colletotrichum coccodes*, registered under the name Velgo® in Canada and the US is considered a successful bioherbicide using a *Colletotrichum* sp. as active ingredient (Templeton, 1992b; Mortensen, 1988; Dauch *et al*., 2002).
Colletotrichum truncatum Andrus & W. D. Moore has shown promising bioherbicide potential against the weed Sesbania exaltata (Raf.) Cory (Silman et al., 1993; Connick et al., 1996). Corn flour-formulations of C. truncatum micro-slerotia incorporated into potting soil enhanced disease in emerging sesbania seedlings (Jackson et al., 1996). Biological control of common St. Johnswort (Hypericum perforatum), an aggressive weed widely disseminated, can be enhanced using a host-specific strain of the fungus, C. gloeosporioides f. sp. hypericum (Penz.) Penz. and Sacc. (Morrison et al., 1998). Colletotrichum gloeosporioides f. sp. malvae was submitted for review by Encore Tech. to the EPA (US Environmental Protection Agency) for possible registration in the US under the name Mallet W. P. but approval is still pending (G. Hnatowich, pers. comm.). Results with the use of C. gloeosporioides for control of lodgepole pine dwarf mistletoe (Arceuthobium americanum Nutt. ex Engelm.) are encouraging, and a worldwide patent is being sought (IBG, 2000). In China, biocontrol with a native fungal strain of C. gloeosporioides f. sp. cuscutae led to the commercialization of LuBao, in 1963, for the control of dodder (Cuscuta sp.) a parasitic weed in soybean. An improved formulation Lubao 2 is still in use today (Wan and Wang, 2001). Other potential uses of Colletotrichum spp. as a mycoherbicide have been reported from many other regions of the world (Sharon, 1998; Nof et al., 1998; Gressel et al., 1998; Killgore et al., 1999). Although many strains of Colletotrichum demonstrated adequate potential in field tests, they have not been developed as commercial products (Templeton, 1992b).

1.4.5. Mating systems in Colletotrichum spp.

Reproduction in most Colletotrichum spp. is vegetative. The sexual perfect stage has not been found in nature or in culture for many species. This is surprising given the
variability of members of the genus (Bryson et al., 1992). The unusual complex mating systems of *Glomerella* spp., ranging from self-fertile (homothallism), self-sterile but cross-fertile (heterothallism) and asexual strains may be correlated with its morphological instability (Bryson et al., 1992). Hence the genus *Glomerella* seems to be unique among the ascomycetes because a wide set of different mating types occur within strains and multiple mating types among the heterothallic strains occur (Hanau et al., 1998).

In *Glomerella cingulata* and *G. graminicola* both homothallic and heterothallic strains exist (Vaillancourt et al., 2000). Homothallic strains of *G. cingulata* give rise to progeny with homothallic, heterothallic reproduction and also sterile progeny (Wheeler, 1954). *G. graminicola* mating systems include true heterothallism and also unbalanced heterothallism and true homothallism (Vaillancourt and Hanau, 1999; Vaillancourt et al., 2000). Most homothallic and heterothallic strains are fertile and homothallic strains frequently give rise to heterothallic progeny (Vaillancourt et al., 2000).

Extensive research in the 1940s and 1950s on the reproduction of *G. cingulata* emphasized an unbalanced heterothallic mating system that resulted from mutation in the morphogenetic pathway necessary to homothallism (Chilton et al. 1944a, b; 1949a, b; Edgerton et al., 1945; Wheeler, 1950; Hanau et al., 1998). In *G. cingulata*, a homothallic culture originated from a single ascospore produced asci containing four self-fertile and four self-sterile ascospores (Perkins, 1987). The new self-fertile ascospores germinate to form self-fertile colonies that give rise to asci containing once more four self-fertile and four self-sterile ascospores. Colonies originating from the self-sterile ascospores apparently mate with the self-fertile strains to form hybrid perithecia (Perkins, 1987). The
molecular basis of this unidirectional shift from self-fertile to self-sterile is not well understood (Glass and Kuldau, 1992).

Isolates of *C. graminicola* can be heterothallic and homothallic, but the homothallism may be a consequence of mutation and selection during repeated subculture and *C. graminicola* may be heterothallic in nature (Vaillancourt and Hanau, 1991). Conidial anastomosis between *C. gossypii* var. *cephalosporioides* isolates as well as sexual reproduction has been reported (Roca et al., 2004a). An experiment conducted with nitrate non-utilizing heterokaryon mutants from field isolates of *C. destructivum*, two *C. gloeosporioides* strains, *C. gloeosporioides* f. sp. *jussiae*, *C. fragariae*, *C. malvarum* and *C. trifolium*, demonstrated the self-compatibility between all the heterokaryons except between *C. destructivum* and *C. fragariae* (Brooker et al., 1991).

**1.4.6. Recombinant Colletotrichum spp.**

Conidial anastomoses between isolates of two different *Colletotrichum* spp. (*C. lindemunthinum* and *C. gossypii*) has been reported (Roca et al., 2004b). The growth of this hybrid was assessed to be less than the parental strains, however, in field conditions natural selection would ensure propagation of any hybrid with a selective advantage (Roca et al., 2004b). Recombination between different lineages of *C. graminicola* from maize and *C. graminicola* from *Pooideae* has also been suggested based on molecular data (Crouch, 2006). Heterokaryons from *C. gloeosporioides* although exhibiting a slow growth rate, were stable and could be perpetuated on minimal media (Chacko et al., 1990). ‘*The potential for sexual reproduction and gene flow within and between genetic subgroups of C. acutatum sensu lato and the influence on population structure remains largely unexplored*’ (Guerber et al., 2003).
The commercialized mycoherbicide Collego®, formulated with *C. gloeosporioides* f. sp. *aeschnomene* (pathogenic to northern jointvetch) can mate, *in vitro*, with other strains of *C. gloeosporioides* pathogenic to different hosts [winged water primrose, *Ludwigia* (*Jussiaea*) *decurrens* and pecan, *Carya illinoensis*] being capable of producing viable, recombinant progeny with variable pathogenicity to apple fruits (Cisar *et al*., 1994). Parental host specific strains from northern jointvetch and pecan crossed on the surface of northern jointvetch plants (in laboratory conditions), producing viable progeny pathogenic only to apples (Cisar *et al*., 1996). Pecan and apple are both affected by *G. cingulata* which causes pecan anthracnose and bitter-rot of apple (Rand, 1914).

Studies of heterokaryosis may indicate the potential for genetic exchange between related subspecies of *Colletotrichum* following field release of mycoherbicides (Chacko *et al*. 1989, 1990). Vegetative compatibility among heterokaryon auxotrophic and nitrate nonutilizing mutant isolates has been reported from *C. orbiculare* (Wasilwa *et al*., 1991), *Gromerella graminicola* (Vaillancourt and Hanau, 1994) and from *C. gloeosporioides* f. sp. *aeschnomene* (Chacko *et al*., 1990). In imperfect asexual stages of fungi, such as *C. gloeosporioides*, heterokaryosis is likely to be a relevant factor in the rapid adaptation of the pathogen to a new host (Sreenivasaprasad *et al*., 1993).

The environmental implications associated with gene flow between a genetically engineered mycoherbicide and endemic fungi have not been determined (TeBeest and Cisar, 1994; Cisar and TeBeest, 1995). Assessment of release risk for these agents must include determination of the likelihood and consequences of gene flow between bioherbicides and related fungi (Teng and Yang, 1993). Positive and negative aspects must be balanced. Genetic manipulation may be useful to enhance the virulence of a
pathogen without a narrow host range or to reduce the host range of a highly virulent pathogen (Kistler, 1991; Wilson and Lindow, 1993).

1.4.7. Infection process

The pathogenicity of Colletotrichum spp. to plants is intrinsically linked with a sequence of a physiological and morphological chain of events (Dickman, 1998). After conidial attachment, germination and development of a germ tube, Colletotrichum spp. penetrates the host through natural openings (stomata), wounds or by direct penetration of the cuticle (Bailey et al., 1992). A highly specialized infection peg called an appressorium allows them to penetrate a healthy host (Emmett and Parbery, 1975; Sutton, 1968; Dean, 1997). The appressorium has a turgor-based mechanical force that mediates the direct penetration of a narrow peg through the host cuticle and epidermal layer aided by the induction of extracellular hydrolitic enzymes that break the polymeric defenses of the host (Money, 1995; Mendgen et al., 1996; Kolattukudy et al., 1998). After hyphal penetration of an epidermal cell, intracellular infection vesicles that produce large primary hyphae develop using nutrient assimilation within the plant tissue. These ramify throughout the apoplast of the leaf tissue, producing secondary hyphae that move throughout the plant via acervuli and are responsible for the shift from biotrophic to necrotrophic infection growth (Dickman, 1998; Wei et al., 2004). The pressure exerted at the tip of the penetration peg of C. graminicola has been estimated at 17 micro Newtons (µN) (Bechinger et al., 1999). To better visualize the exceptional force exerted by these structures a 17µN pressure exerted by a human hand would allow it to support an 8-ton school bus (Money, 1999a) or a whale (Money, 1999b). The pressure exerted by an
infection peg of the fungus *Magnaporthe grisea* has been estimated to be higher than any other living organism (Howard *et al*., 1991).

*Colletotrichum* spp. have different infection strategies, as follows: (1) intracellular hemibiotrophs, the fungus grows initially biotrophically and no symptoms are produced on the plant, followed by a visible destructive phase in which the pathogen becomes necrotrophic; (2) subcuticular intramural pathogens that are characterized by subcuticular growth that dissolves the pectic matrix of the epidermis cell walls, and (3) species exhibiting both hemibiotrophic and subcuticular intramural infections (Smith *et al*., 1999). Strict hemibiotrophs have non-overlapping biotrophic and necrotrophic fungal structures formed in different tissues of the plant. These hemibiotrophs are often found in *Colletotrichum* spp., but rarely in other fungi such as *Phytophthora infestans* (Oliver and Ipcho, 2004). The infection process of *C. graminicola* on different Poaceae hosts has been extensively evaluated. The following sequence of events was observed: spore germination occurs 2 h after inoculation, appressoria germinate within 6 h, cuticule penetration takes place 8 h after inoculation and infection hyphae develop inside the epidermal cells within 24 h (Perfect and Green, 2001; Khan and Hsiang, 2003; Wei *et al*., 2004).

Fungal pathogenicity has been widely studied through different techniques (Gold *et al*., 2001). Different compounds and enzymes, produced by the host and pathogen, *Colletotrichum* spp., have been isolated (Bailey *et al*., 1992; Dean, 1997; Kubo, 1998; Prusky *et al*., 1998; Hutchison *et al*., 2000). Phytoplane microflora (Slade *et al*., 1986; Jeffries, 1998), pH (Yakoby *et al*., 2000; Prusky *et al*., 2002), nitrogen sources (Drori *et al*., 2003) and genes expressed during infection have been studied and known to be
implicated in triggering appressoria formation, host infection and pathogenesis development (Takano et al., 2000; Dickman, 2004).

1.4.8. Quiescent infection by Colletotrichum spp.

The role of endophytic growth of fungi in plants has been associated with their symbiotic activity with the plant host. Endophytes may have antagonistic capabilities to other plant pathogens (i.e., fungi and bacteria) and insect plant pests, as well as, the ability to enhance the physiological activities of the plant host (e.g., drought tolerance and/or growth increase) (Azevedo et al., 2000).

Endophytic Colletotrichum spp. often penetrates leaves directly or enters through stomata openings and then ramifies to the mesophyll intercellularly, showing no symptoms of infection (Verhoeff, 1974; Carrol, 1995; Wei et al., 1997; Latunde-Dada, 2001). A physiological change, such as senescence of the host will trigger the necrotic phase of the fungus and the occurrence of visible necrosis. The quiescent (or latent) infection occurs in tropical and subtropical fruits in their early stages of development with symptoms appearing only in the near-mature or mature stage of fruit development (Kuo, 1999). This occurs due to adverse physiological conditions temporarily imposed by the host (Prusly and Pumbley, 1992). Thus, appressoria do not germinate but the tissues are instead indirectly penetrated by vegetative growth through stomata (Latunde-Dada, 2001). After the fungus senses the substrate, a process associated with conidial adhesion and appressorium differentiation (Hoch, 2004) involving chemical signals from the host (in its senescence or reproductive stage) will trigger the infection process through the germination of appressoria (Kolattukudy et al., 1998; Latunde-dada, 2001). It has been suggested that the lifestyle expressed by Colletotrichum spp. (mutualism, parasitism or
commensalism) is controlled by the host plant genotype (Redman et al., 2001). Also, it has been reported that both chemical and physical stimuli between the fungus and plant eventually alter gene expression, producing a cellular response (Memmott et al., 2002). Experimental evidence reported by Kolattukudy et al. (1998) suggests that ‘conidia of C. gloeosporioides are primed by a 2h contact with a hard surface that induces a set of genes; this priming enables the conidia to respond to host signals such as host surface wax and the host ripening hormone, ethylene, which induce another set of genes that enables them to germinate and differentiate into appressoria’. These signals are released from the host and pathogen and determine the type of symbiotic relationship that will develop (Dickman 2000, 2004).

Methods to trace latent infection and gene expression by Colletotrichum spp. have been developed. These are: DNA extraction and Polymerase chain reaction (Parikka and Lemmetty, 2004) and green fluorescent protein-transgenic strains (Dumas et al., 2001; Horowitz et al., 2002). Several Colletotrichum spp. have been reported to have mutualistic or comensalistic lifestyles in plants. Colletotrichum spp. that have an endophytic behavior are C. cocoodes, on tomato (Dillard and Cobb, 1998) and potato (Tso and Johnson, 2000), C. musae on banana and C. gloeosporioides on several plant hosts such as cowpea and citrus (Zulfiqar et al., 1996; Cerkauskas, 1988; Latunce-Dada, 2001), C. demantium f. sp. truncatum, on soybean (Sinclair, 1991), C. gloeosporioides f. sp. malvae on 10 different field crops (Makowski and Mortensen, 1998) and C. acutatum on strawberry, pepper, egg-plant, etc. (Freeman et al., 2001b; Horowitz et al., 2002; Zulfiqar et al., 1996).
Studies with *Colletotrichum magna* mutants showed that nonpathogenic lifestyles provide a broader host range than the parental pathogenic *C. magna* strains and that nonpathogenic mutants conferred an array of mutualistic benefits on the host plant (e.g., resistance against wild type *Colletotrichum* spp. and drought, growth enhancement and biomass increase) (Redman *et al*., 2001).

### 1.4.9. Survival and dispersal under different abiotic conditions

Asymptomatic plants that are not considered hosts of *C. acutatum*, living symbiotically or non-pathogenically, may serve as a potential focal source for infection. These allow the pathogen to survive between growing seasons of the primary plant host species (Freeman *et al*., 2001b).


It has been determined that conidia of *C. acutatum* in an infected leaf showed a decreased survival rate under moist or saturated conditions and increased survival (maintaining pathogenic potential) under dry conditions, living up to 12 months after being incorporated into sandy soil with a pH of 6.9 (Norman and Strandberg, 1997). Overwintering survival of *C. acutatum* from non-mummified strawberry fruits located on
and below the soil surface have been also reported (Wilson et al., 1992) as well as in high bush blueberry, *Vaccinium corymbosm* (DeMarsay and Oudemans, 2001). *C. acutatum* conidial spores are more tolerant to extended periods of low temperatures than *C. gloeosporioides*, where spore germination, survival and appressoria formation can be compromised (Everett, 2003). Survival in the soil may be enhanced by low temperatures or greater burial depths (Eastburn and Gubler, 1990). Summer survival of *C. acutatum* has been determined to be lower (4%) than *C. gloeosporioides* (96%) after soil burial of infected strawberry crowns (Ureña-Padillo et al., 2001). *Colletotrichum acutatum* was not recovered after 56 d of summer burial; *C. gloeosporioides* recovery was 10-20% after 70 d, suggesting that oversummering crop debris did not serve as inoculum for epidemics of *Colletotrichum* spp. crown rot (Ureña-Padillo et al., 2001).

*Colletotrichum coccodes* survives longer as free sclerotia in the soil than associated with plant tissue. After eight yr the viability of free sclerotia was 0.0, 90 and 80% at the soil surface, 10 and 20 cm soil depth, respectively (Dillard and Cobb, 1998). This may result from greater fluctuation in temperature and moisture at the soil surface (Dillard and Cobb, 1998). Sporogenic germination of sclerotia originated from *C. coccodes*, occurred at a wide range of temperatures, with production of conidial masses from 10-34°C in aerated plates, *in vitro* (Sanogo and Pennypacker, 1997).

Sclerotium are important dormant or quiescent structures of many plant pathogens, being extremely resistant to harsh conditions (i.e., desiccation, low temperatures, fungicides, etc.), enabling fungal pathogens to overcome adverse periods and germinate when conditions improve (Coley-Smith and Cooke, 1971). They originate initially from a cluster of hyphae - a mass of tightly interwoven hyphal cells - then
differentiate into external thick-walled and melanized hyphae and internal extended vacuolated hyphae. The inner tissue is nutrient rich with abundant lipid and glycogen reserves surrounded by a cortex of cells with thicker walls (Tu, 1980; Carlisle and Watkinson, 1997).

1.5. Research objectives

The present research intends to contribute to a better understanding of the etiology of fungal epizootics in general and of Colletotrichum in particular, through the study of the natural occurring epizootic in populations of F. externa in the northeastern hemlock forests. The main objectives of this research are:

1. To identify the causal agents of the emergent epizootic within populations of the northeastern elongate hemlock scale, Fiorinia externa, by molecular and morphological approaches, as well as its geographic distribution;

2. To characterize morphologically, biologically and phylogenetically the Colletotrichum sp. strain commonly isolated from areas of the epizootic;

3. To assess the virulence of the ubiquitous Colletotrichum sp. strain present in the geographic range of the epizootic to four families of insects and five families of plants.
Table 1.1. Reports of fungal species recovered from populations of scale insects

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Host scale</th>
<th>Location</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aschersonia turbinata</em> Berk.</td>
<td>Wax scale (<em>Ceroplastes floridensis</em> Comstock)</td>
<td>Florida (USA)</td>
<td>a,b</td>
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<tr>
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<td>Liriodendron scale [<em>Toumeyella liriodendri</em> (Gmelin.)]</td>
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<tr>
<td></td>
<td>Tessellated scale (<em>Eucalymnatus tessellates</em>)</td>
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<tr>
<td><em>Aschersonia alepyrodis</em> (Webber)</td>
<td>West Indian Red scale [<em>Selenaspis articulatus</em> (Morgan)]</td>
<td>Brazil</td>
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<td></td>
<td><em>Parlatoria ziziphus</em> (Lucas)</td>
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<tr>
<td><em>Aschersonia basicystis</em> Berk. &amp; MA Curtis.</td>
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<tr>
<td><em>Aschersonia placenta</em> Berk. and Br.</td>
<td><em>Asterolecanium ungulata</em> Russel</td>
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<td></td>
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<td>India</td>
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<tr>
<td><em>Aschersonia duplex</em> Berk.</td>
<td><em>Metaceronema japonica</em> Mask.</td>
<td>China</td>
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<tr>
<td><em>Hypocrella duplex</em> (Berk.)</td>
<td>Coccoidea</td>
<td>Caucasus</td>
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<td></td>
<td><em>Parlatoria ziziphus</em> (Lucas)</td>
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<tr>
<td><em>Aschersonia sp.</em></td>
<td>Japanese scale (<em>Lopholeucapsis japonica</em> Cockerell)</td>
<td>Georgia (Caucasus)</td>
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<td></td>
<td>Brown scale [<em>Saissetia coffeae</em> (Walker)]</td>
<td>India</td>
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</tr>
<tr>
<td><em>Hypocrella turbinata</em> (Berk.)</td>
<td>Wax scale (<em>Ceroplastes floridensis</em> Comstock)</td>
<td>Florida (USA)</td>
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<td>Petch</td>
<td>Purple scale [<em>Lepidosaphes beckii</em> (Newm.)]</td>
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<td>Soft brown scale (<em>Coccus hesperidum</em> Linn.)</td>
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<tr>
<td><em>Hypocrella javanica</em> (Penz. and Sacc.)</td>
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<td>Unidentified scales</td>
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<td></td>
<td><em>Parlatoria</em> sp.</td>
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<td><em>Hypocrella raciborskii</em></td>
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<td><em>Hypocrella palmae</em> (Berk. &amp; MA Curtis) Sacc.</td>
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<td>Caucasus</td>
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(Continue)
Table 1.1 (Continued). Reports of fungal species recovered from populations of scale insects

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Host scale</th>
<th>Location</th>
<th>Source</th>
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<td>Hirsutella lecaniicola (Jaap)</td>
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<tr>
<td>Hymenostilbe lecaniicola (Jaap) Mains</td>
<td>Coccoidea</td>
<td>USA</td>
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<td></td>
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<td>Canada</td>
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<td>Romenia</td>
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<tr>
<td>Myriangium duriae Mont.</td>
<td>Ischnaspis filiformis Douglas</td>
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<tr>
<td>Myriangium floridanum Hoehn.</td>
<td>Snow scale (Chionaspis citri Comst.)</td>
<td>Florida (USA)</td>
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<td>Purple scale (Lepidosaphes beckii (Newm.)</td>
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<td>Chaff scale (Parlatoria pergandii Comst.)</td>
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<td>Myriangium montagnei Berk.</td>
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<td>Myriangium asterinosporum (Ellis &amp; Everh.)</td>
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(Continue)
### Table 1.1 (Continued). Reports of fungal species recovered from populations of scale insects

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<td><em>Lecanium mangiferae</em> Green</td>
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<td><em>Chephalosporium lecanii</em> Zimmermann</td>
<td><em>Diaspis</em> sp.</td>
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<td>San José Scale (<em>Quadraspidiotus perniciosus</em> Comstock)</td>
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<td>Purple scale [<em>Lepidosaphes beckii</em> (Newm.)]</td>
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<td>Chaff scale (<em>Parlatoria pergandii</em> Comst.)</td>
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<td><em>Sphaerostilbe flammea</em> Tul.</td>
<td><em>Chionaspis citri</em> Comst.</td>
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<td><em>Sphaerostilbe aurantiicola</em>  (Kerk. and Br.)</td>
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<td><em>Podonectria aurantii</em> (V. Höh) Petch</td>
<td>Citrus scales</td>
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<td>Black parlatoria scale [(<em>Parlatoria zizyphus</em> (Lucas)]</td>
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<td><em>Podonectria coccicola</em> (Ellis and Everh.) Petch</td>
<td>Chaff scale (<em>Parlatoria pergandii</em> Const.)</td>
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(Continue)
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<td><em>Aspidiotus</em> sp.</td>
<td>Algeria</td>
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<td>Oystershell scale</td>
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<td><em>Fusarium</em> sp.</td>
<td><em>Dictyospernum</em> scale</td>
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<td><em>Fusarium coccophilum</em> (Desm.)</td>
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<td>Caucasus</td>
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<td>Wollenweber &amp; Reinking</td>
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<td><em>Fusarium microcera</em> Bilai</td>
<td>Coccoidea</td>
<td>Soviet Union</td>
<td>y</td>
</tr>
<tr>
<td>[telemorph: <em>Calonectria decora</em> (Wallr.) Sacc.]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Torrubiella lecanii</em> Johnston</td>
<td>Hemispherical scale [<em>Saissetia hemisphaerica</em> (Targ.)]</td>
<td>Cuba</td>
<td>d</td>
</tr>
<tr>
<td>Pegiotrichum saccarinum Raw.</td>
<td>Unidentified scale</td>
<td>Brazil</td>
<td></td>
</tr>
<tr>
<td><em>Acrostalagmus albus</em> Pr.</td>
<td>Soft brown scale (<em>Coccus hesperidum</em> Linn.)</td>
<td>Italy</td>
<td></td>
</tr>
<tr>
<td><em>Isaria</em> sp.</td>
<td>Black scale [<em>Saissetia oleae</em> (Bern.)]</td>
<td>North Carolina</td>
<td>o</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Virginia</td>
<td></td>
</tr>
</tbody>
</table>

Sources:  

- a – Webber (1894, 1897)  
- b – Berger (1921)  
- c – Fawcett (1944)  
- d – Fawcett (1948)  
- e – Parkin (1906)  
- f – Watson and Berger (1937)  
- g – South (1910)  
- h – Rolfs (1897)  
- i – Rolfs and Fawcett (1908)  
- j – Kuwana *et al.* (1904)  
- k – Balazy (1999)  
- l – Annecke (1963)  
- m – Rao and Sohi (1980)  
- n – Wolcott (1940)  
- o – Charles (1941)  
- r – Lim *et al.* (1991)  
- s – Ibrahim and Kon (1992)  
- t – Devnath (1986)  
- u – Shiryaeva (2000)  
- v – Zhuan (1988)  
- w – Das and Ganguli (1961)  
- x – Evlachova (1974)  
- y – Koval (1974)  
- z – Borisov *et al.* (2001)
### Table 1.2. Reports of fungi recovered from **Fiorinia** species

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Host Scale</th>
<th>Location</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sphaerostilbe coccophilus</em></td>
<td><em>F. fioriniae</em></td>
<td>Ceylon</td>
<td>a</td>
</tr>
<tr>
<td><em>Nectria diploa</em> Berk. and Curt.</td>
<td><em>F. rubrolineata</em></td>
<td>Ceylon</td>
<td>b</td>
</tr>
<tr>
<td><em>Pyrenochaeta sparsibarba</em></td>
<td><em>F. juniperii</em></td>
<td>Ceylon</td>
<td>b</td>
</tr>
<tr>
<td><em>Muricularia calva</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Stilbum coccorum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium sp.</em></td>
<td><em>F. thea</em></td>
<td>India</td>
<td>c</td>
</tr>
</tbody>
</table>

Sources:
- a – Parkin (1906)
- b – Petch (1922)
- c – Das and Das (1962)
Table 1.3. Sequence of morphological identifications of the most commonly isolated fungus from diseased *F. externa* in the states of Connecticut, New York, New Jersey and Pennsylvania.

<table>
<thead>
<tr>
<th>Year</th>
<th>Institution</th>
<th>Identifier</th>
<th>ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>2003</td>
<td>Entomology Research Laboratory, University of Vermont, USA</td>
<td>Drs. Svetlana Gouli and Vladimir Gouli</td>
<td><em>Hypocrella</em> sp.</td>
</tr>
<tr>
<td>2005</td>
<td>Mycological Society of China, China</td>
<td>Dr. Zengzhi Li</td>
<td><em>Aschersonia marginata</em></td>
</tr>
<tr>
<td>2005</td>
<td>Cornell University, USA Systematic Botany and Mycology Laboratory</td>
<td>Dr. R. Humber, Dr. A. Rossman</td>
<td><em>Fusarium merismoides</em></td>
</tr>
<tr>
<td>2005</td>
<td>Cornell University, USA Cornell’s Plant Pathology Herbarium</td>
<td>Dr. Kathie T. Hodge</td>
<td><em>Tubercularia</em> sp.</td>
</tr>
<tr>
<td>2006</td>
<td>National Center for Genetic Engineering and Biotechnology, Thailand</td>
<td>Dr. Nigel Hywel-Jones</td>
<td><em>Myriangium</em> sp.</td>
</tr>
</tbody>
</table>
CHAPTER 2

Fungi Associated with a Natural Epizootic in *Fiorinia externa* Ferris (Hemiptera: Diaspididae) populations

José A. P. Marcelino, Svetlana Gouli, Rosanna Giordano, Vladimir V. Gouli, Bruce L. Parker, Margaret Skinner

**Abstract:** Stands of eastern hemlock in the northeastern United States are in decline, in part from the activity of elongate hemlock scale, *Fiorinia externa* Ferris (Hemiptera: Diaspididae). From 2001 to the present a natural epizootic has been observed in populations of *F. externa*. Initially discovered at the Mianus River Gorge Preserve in Bedford, NY, the epizootic also has been detected in Pennsylvania, New Jersey and Connecticut. Understanding and assessing the identity of the pathogenic microorganisms responsible for this natural mortality is crucial for developing biological controls for this pest. We have isolated and taxonomically and genetically identified entomopathogens, phytopathogens and endophytic fungi associated with *F. externa*. Isolates of the following were obtained: *Colletotrichum* sp., *Lecanicillium lecanii*, *Beauveria bassiana*, *Cordyceps* sp. anamorph, *Myriangium* sp., *Mycosphaerella* sp. anamorph, *Nectria* sp., *Botrytis* sp., *Phialophora* sp. and *Fusarium* sp.

**Keywords:** epizootic, biological control, entomopathogenic fungi, *Tsuga canadensis*

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Acknowledgments: We thank Lora Schwarzberg (New York State Department of Environmental Conservation) and Teri Hata (University of Vermont, Entomology Research Laboratory) for valuable help collecting specimens and for technical assistance. We also thank Drs. David TeBeest (University of Arkansas) and Roberto Cesnik (EMBRAPA, Brazil) for their scientific input and insights. This work was funded in part through a grant awarded by the Northeastern Area State and Private Forestry, USDA Forest Service (# 04-CA-11244225286) and is in partial fulfillment of requirements for the PhD degree of J.M. at the University of Vermont.
Evidence of significant decline and death of eastern hemlocks \([Tsuga canadensis (L.) Carrière]\) has been observed in the northeastern United States. It has been attributed in part to feeding by the elongate hemlock scale, \(Fiorinia externa\) Ferris (Hemiptera: Diaspididae) and the hemlock woolly adelgid, \(Adelges tsugae\) Annand (Hemiptera: Adelgidae) (Colbert et al. 2002, Danoff-Burg and Bird 2002). \(Fiorinia externa\) is found along the eastern seaboard from Massachusetts to North Carolina. Populations reach as far west as Ohio, with the highest density of infestation occurring within a 300 km radius of New York City (USDA Forest Service 2004). Natural biological control agents have not succeeded in keeping populations of \(F. externa\) below damaging levels. Insecticide treatment of this pest has also proved ineffective as populations resurge rapidly after application (McClure and Fergione 1977). High parasitism rates of \(F. externa\) by the aphelinid parasitoid, \(Encarsia citrina\) Craw (\(Aspidiotiphagus citrinus\) Howard) (Hymenoptera: Aphelinidae) have been observed in its native region of Japan (McClure 1986), but this species does not occur in the northeastern United States (McClure 1977). The armored shield of \(F. externa\) helps to protect it from insecticides, natural enemies and adverse weather conditions.

Given the rapid decline of hemlock, it is imperative that control methods are devised that are ecologically sound and cost effective. In 2001 an epizootic within populations of \(F. externa\) was discovered in the Mianus River Gorge Preserve in Bedford, NY, where 13-27% of sampled insects were covered by profuse sclerotic masses which often concealed the insects’ body (McClure 2002). Forested areas with similarly infected scales have been found in several counties in New York, Pennsylvania, Connecticut and New Jersey (Parker et al. 2005).
To evaluate the role of epizootics in the suppression of insect pests, it is essential to determine the causal agent(s) involved in the disease. The objective of this study was to morphologically characterize and molecularly identify the mycobiota associated with the epizootic of *F. externa*.

**Materials and Methods**

**Sampling**

Thirty-six sites were surveyed and collections of infected *F. externa* adults (i.e., with sclerotic masses or other visible signs of mycoses) were made from 2004 to 2006 in New York (nine counties), Pennsylvania (three counties), Connecticut (two counties) and New Jersey (one county) (Figure 2.1). Surveys were made in areas with a high percentage of hemlock trees, based on land cover maps from the NY Dept. of Environmental Conservation, and in hemlock stands in state and county parks, camping areas, city-owned reservoirs, forest preserves, sanctuaries, historic sites and arboretums. In each hemlock stand, a minimum of 100 hemlocks were randomly sampled, taking five 40-cm long branches per tree, from which to obtain infected scales and isolate fungi. In 10 sites in New York and four in Pennsylvania, a sub-sample of 10 twigs from five branches was used to make a visual estimate of the percentage of *F. externa* showing evidence of fungal infection, i.e., sclerotic masses on the scale surface, abnormal pigmentation and loss of turgor or mycelium arising from the body of the insect.
Figure 2.1. Geographical distribution of sites with diseased *Fiorinia externa* populations (2002-2006).
**Isolation of fungi**

Scale-infested twigs were inspected under a stereomicroscope, and those with signs of mycosis were surface sterilized by dipping in a solution of 2.5% sodium hypochlorite with 0.1% Silwet L-77 for 45 s, rinsed with sterile distilled water (SDW) and placed on potato dextrose agar (PDA) supplemented with Penicillin (5 ml/l) and Streptomycin (12.5 ml/l). Other selective media were also used for isolation of fungi: Sabouraud dextrose agar and yeast, Sabouraud dextrose agar supplemented with egg yolk and milk, bacto-agar with beef extract, bacto-agar with liver extract, and modified media supplemented with Schneider’s insect medium (Sigma Chemical Co., St. Louis, MO). All isolates were purified using standard dilution techniques (Schmitthenner and Hilty 1962), from which monosporic lines were generated for the most commonly retrieved fungi, using a stage-mounted DC3001 micromanipulator (World Precision Instruments, Inc., Sarasota, FL).

**Molecular identification of fungi**

DNA of the most commonly isolated fungi was extracted from one-week-old cultures of the fungi isolated from the pest, from sclerotia and also *F. externa* enclosed in sclerotia (Table 2.1).
Table 2.1. Pure fungal isolates recovered from *Fiorinia externa*

<table>
<thead>
<tr>
<th>Fungal group</th>
<th>Species/Genus</th>
<th>Geographic origin</th>
<th>Year Collected</th>
<th>Reference code</th>
<th>DNA Extraction from</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Entomopathogens</strong> (Σ= 121)</td>
<td><em>Myriangium</em> sp.</td>
<td>Bayberry Lane, NY *</td>
<td>2006</td>
<td>CEHS206</td>
<td>1. Culture</td>
<td>EF464574 EF464585</td>
</tr>
<tr>
<td>(N= 62 isolates)</td>
<td></td>
<td>Litchfield, CT</td>
<td></td>
<td>SEHS206</td>
<td>2. Sclerotia</td>
<td>EF464575 EF464586</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Litchfield, CT</td>
<td></td>
<td>SINEHS206</td>
<td>3. Sclerotia</td>
<td>EF464576 EF464587</td>
</tr>
<tr>
<td></td>
<td><em>Cordyceps</em> sp. anamorph</td>
<td>Valley Forge, PA</td>
<td>2005</td>
<td>CEHS133a</td>
<td>1. Culture</td>
<td>EF464571 EF464589</td>
</tr>
<tr>
<td>(N= 41 isolates)</td>
<td></td>
<td></td>
<td></td>
<td>INEHS133a</td>
<td>2. <em>F. externa</em></td>
<td>EF464572 EF464583</td>
</tr>
<tr>
<td></td>
<td><em>L. lecanii</em></td>
<td>Mount Tom Forest Preserve, MA</td>
<td>2005</td>
<td>EHS132</td>
<td>1. Culture</td>
<td>EF464573 EF464584</td>
</tr>
<tr>
<td>(N= 2 isolates)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>B. bassiana</em></td>
<td>Mount Tom Forest Preserve, MA</td>
<td>2005</td>
<td>EHS163</td>
<td>1. Culture</td>
<td>□</td>
</tr>
<tr>
<td>(N= 16 isolates)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>□</td>
</tr>
<tr>
<td><strong>Phytopathogens</strong> (Σ= 58)</td>
<td><em>Mycosphaerella</em> sp.</td>
<td>Pawling Preserve, NY</td>
<td>2006</td>
<td>EHS201</td>
<td>1. Culture</td>
<td>EF619924 EF619925</td>
</tr>
<tr>
<td>(N=1 isolate)</td>
<td>anamorph</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Colletotrichum</em> sp.*</td>
<td>NY; PA; NJ; CT *</td>
<td>2004/05</td>
<td>EHS48</td>
<td>1. Culture</td>
<td>EF464580 EF464593</td>
</tr>
<tr>
<td>(N= 54 isolates)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Continue)
<table>
<thead>
<tr>
<th>Fungal group</th>
<th>Species/Genus</th>
<th>Geographic origin</th>
<th>Year Collected</th>
<th>Reference code</th>
<th>DNA Extraction from</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phytopathogens</strong></td>
<td><em>Nectria</em> sp.</td>
<td>Valley Forge, PA</td>
<td>2006</td>
<td>EHS144</td>
<td>1. Culture</td>
<td><strong>EF464570</strong> <strong>EF464588</strong></td>
</tr>
<tr>
<td>(∑= 58)</td>
<td>(N= 1 isolate)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Botrytis</em> sp.</td>
<td>Bayberry Lane, NY</td>
<td>2006</td>
<td>EHS265</td>
<td>1. Culture</td>
<td>□ □</td>
</tr>
<tr>
<td></td>
<td>(N= 1 isolate)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Fusarium</em> sp.</td>
<td>South Salem, NY</td>
<td>2006</td>
<td>EHS290</td>
<td>1. Culture</td>
<td>□ □</td>
</tr>
<tr>
<td></td>
<td>(N= 1 isolates)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Endophyte</strong></td>
<td><em>Phialophora</em> sp.</td>
<td>South Salem, NY</td>
<td>2006</td>
<td>EHS291</td>
<td>1. Culture</td>
<td><strong>EF464577</strong> <strong>EF464590</strong></td>
</tr>
<tr>
<td>(∑= 1)</td>
<td>(N= 1 isolate)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Counties: New York (Columbia, Dutchess, Kings, Orange, Putnam, Queens, Rockland, Ulster and Westchester); Pennsylvania (Bucks, Chester and Northampton); New Jersey (Passaic); Connecticut (Fairfield and Litchfield).

* Origin of samples for use in DNA extraction.

□ Only morphological identification was performed.
The Power Soil™ DNA kit (Mo Bio Laboratories, Inc., Carlsbad, CA) was used according to the manufacturers’ instructions with the following exceptions: 1) Samples were shaken for 5 min at 5.5 m/s to facilitate opening of the fungal cell walls using a FastPrep™ FP120 machine (Thermo Savant, Holbrook, NY); and 2) DNA was eluted using 100 µl of diluted elution buffer (1:15) (Quiagen, Valencia, CA) and concentrated to 20 µl with a speed vacuum (Eppendorf Centrifuge 5415C, Vaudaux, Switzerland) prior to downstream applications. Polymerase Chain Reaction (PCR) was performed using Ready-To-Go RT-PCR beads (Amersham Biosciences Inc., Piscataway, NJ). The D1/D2 region of the 28S ribosomal DNA commonly used for phylogenetic analysis at the genus level and above (Hillis and Dixon 1991) was amplified with primers NL1 and NL4 (O’Donnell 1992, 1993).

The internal transcribed spacers (ITS) variable region was amplified using primers ITS1 and ITS4 (White et al. 1990) for within-species differentiation (Talhinhas et al. 2002, Afanador-Kafuri et al. 2003). The 28S ribosomal DNA gene was amplified using the following protocol: initial denaturation at 95° C for 2 min followed by 30 cycles of 95° C for 30 s, annealing at 50° C for 30 s and elongation at 72° C for 1 min. The protocol used for the amplification of the ITS region was the same as above, except that the annealing temperature was raised to 52° C. PCR products were purified using the QIAquick® PCR purification kit (Qiagen, Valencia, CA) or Centri Spin™ columns (Princeton Separation, Adelphia, NJ). DNA was stored at 4° C. PCR products were sequenced using BigDye v1 (Applied Biosystems, Foster City, CA) according to the following protocol: initial denaturation at 95° C for 3 min followed by 30 cycles of 95° C for 10 s, annealing at 50° C for 5 s, and elongation at 60° C for 2 min.
Sequencing reactions were run on a 3130xl Genetic Analyzer (Applied Biosystem, Foster City, CA). Chromatograms were examined, and contiguous sequences were generated, using Sequencher™ (Gene Codes Corporation, Ann Arbor, MI). Sequences generated from this study were compared to related sequences in GenBank® using BLAST (Altschul et al. 1990). Matches obtained from these searches were checked against our preliminary morphological identifications. Sequences obtained for this study were deposited in GenBank® (accession numbers included in Table 2.1).

Morphological identification

Pure cultures of fungi from *F. externa* were prepared for microscopic morphological identification using the methods of Humber (1997) and contrasted with reference taxonomical guides (Carmichael et al. 1980, Barnett and Hunter 1998, Samson et al. 1988). Scales with superficial signs of mycosis were also prepared for morphological identification of fungal propagules using scotch tape impressions according to the method of Gouli et al. (2005). All fungal isolates were deposited in the entomopathogenic fungal germplasm collection at the Entomological Research Laboratory, University of Vermont, Burlington, VT, USA.

Results and Discussion

In the site where the epizootic was first reported up to 36.8% of the sampled scales were partially or completely covered with sclerotia in 2004. These sclerotia were usually brown, irregular masses when associated with the new generation of infected scales and turned black after several months (Figure 2.2).
Figure 2.2. *Fiorinia externa* showing typical symptoms of fungal infection, and representative propagules from several entomopathogenic fungi found associated with *F. externa* in epizootic areas. A. Scale covered with a fresh fungal sclerotium; B. Old, weathered sclerotia covering scale adults; C. *Myriangium* sp. hyphae (CEHS208); D. *Beauveria bassiana* mycosis; E. *Beauveria bassiana* conidia (EHS163) F. *Lecanicillium lecanii* (EHS132) mycosis; G. *Lecanicillium lecanii* conidia; H. *Cordyceps* sp. anamorph mycosis; I. *Cordyceps* anamorph sp. conidia (CEHS133a); J. *Colletotrichum* sp. conidia (EHS48); K. *Fusarium* sp. conidia (EHS290); L. *Mycosphaerella* anamorph sp. conidia (EHS201).

Bars: A, B, D, F, H = 0.5 mm; E, G, L = 40 µm; J, K, I, C = 25 µm.
No evidence of fungal hyphae penetrating hemlock needles was detected. The proportion of scales showing signs of fungal infection varied among the different sites. An approximate estimate of the percentage of scales with sclerotic masses in the sites sampled within New York, based on the number of infected scales in the sample stems (10 hemlock twigs/5 branches) averaged ca. 26%, ranging from 61.8% in South Salem, to 2% in Esopus. In Pennsylvania, the proportion of infected scales on the sampled twigs was 9.5% ranging from 27.6% at the Ralph Stover State Park to 0.8% in Jacobsburg. A variety of fungi were cultured and microscopic observations of conidia, conidiophores, mycelium and appressoria made. Molecular analysis using the 28S and ITS sequences facilitated the identification of the genera of the fungi. In many cases molecular analyses confirmed morphological identifications. A total of 180 pure fungal cultures were obtained from infected *F. externa* adults, comprising four entomopathogens, five phytopathogens and one endophytic species. *Myriangium* sp. was molecularly identified with DNA extracted from a pure fungal culture and also from single sclerotia, with no insect body parts, as well as sclerotia with enclosed mummified *F. externa*. *Cordyceps* sp. was molecularly identified with DNA extracted from both a pure fungal culture and *F. externa* adults with signs of mycosis. For all other fungi identified molecularly, DNA was obtained from pure fungal cultures. A total of 20 molecular identifications were obtained from *F. externa* with signs or symptoms of disease (Table 2.1).

**Entomopathogenic Fungi**

A total of 121 fungal isolates known as entomopathogenic species were collected comprising 16 isolates of *Beauveria bassiana*, two *Lecanicillium lecanii*, 41 *Cordyceps* sp. anamorph and 62 isolates of *Myriangium* sp.
The entomopathogenic isolates were among the most well-known and commercially available fungi. For example, *B. bassiana* is known to be pathogenic to 100 insect species (McCoy et al. 1988); *Lecanicillium lecanii* has been used to control coccids, aphids and whiteflies (Feng et al. 2000). *Cordyceps* is primarily a parasite of insects (Nikoh and Fukatsu 2000), while *Myriangium* has proven effective in eradicating populations of scales (Van Epenhuijsen et al. 2000).

*Myriangium* sp. was the most prevalent entomopathogenic fungus isolated from *F. externa*. It was isolated from fresh and old weathered single sclerotia (i.e., portion of sclerotia with no insect body parts) and from sclerotia enclosing *F. externa*. This fungus was not recovered from all of the sample sites, which may be due to the difficulty in isolating this organism, rather than non-occurrence. *Myriangium* spp. are usually exclusive pathogens of scale insects and form a stromata identical to the one present in all the epizootic sites sampled (Miller 1940). Earlier studies described the presence of *Myriangium* spp. on scale insects in Florida but its existence in latitudes other than tropical and sub-tropical is unusual (Fisher et al. 1949). The use of *Myriangium* spp. for management of scale insects has been limited due to the difficulties associated with its culture *in vitro*. Its host specificity makes it an optimal candidate for management of *F. externa*.

**Phytopathogenic and Endophytic Fungi**

Fifty nine phytopathogenic isolates were recovered from *F. externa*: 54 isolates of *Colletotrichum* sp., two *Botrytis* sp., one *Nectria* sp., one *Mycosphaerella* sp. anamorph and one *Fusarium* sp. One endophytic fungal isolate of *Phialophora* sp. was also recovered (Table 2.1).
Given the mode of action of phytopathogenic fungi, it is possible that these isolations are a result of secondary infections in host insects by other pathogens that may have overtaken the immune system of the host. *Colletotrichum* sp., a well-known phytopathogen, was the most prevalent fungus associated with the *F. externa* epizootic and was present in all localities sampled. In addition, this strain was topically applied to *F. externa* in controlled bioassays and recovered after surface sterilization, confirming Koch’s postulates. The above suggests that this *Colletotrichum* strain could have a key role in the epizootic. This genus has been widely associated with phytopathogenicity (Latunde-Dada 2001), not entomopathogenicity. However, *C. gloeosporioides*, has been reported to infect the scale *Orthezia praelonga* Douglas, (Hemiptera: Ortheziidae) on citrus in Brazil (Cesnik and Ferraz 2000) where it is currently under development for commercialization as a myco-pesticide (R. Cesnik pers. comm.). Due to the phytopathogenic activity of this genus, it is critical to evaluate the agent-host-plant interactions (Sands and Van Driesche 2000), and to conduct a comprehensive assessment of its biology and genetics. A detailed molecular characterization of this strain is underway to further evaluate its relationship with other phytopathogenic isolates. *Colletotrichum* sp. from the epizootic areas has been found to have an endophytic association with many locally occurring plants (Marcelino et al. submitted). We hypothesize that due to its ubiquitous presence, the *Colletotrichum* sp. isolated plays a significant role in the dynamics of the epizootic in populations of *F. externa*.

**The Fungal Complex**

It is possible that more than one fungal species is responsible for this epizootic. The virulence and infectivity of pathogens are known primary factors involved in the
initiation and development of epizootics. It is common for epizootiological studies to only consider one pathogen infecting an insect population, though an insect can be affected simultaneously by multiple pathogenic species (Fuxa and Tanada 1987, Tanada and Kaya 1993). Several fungi have been implicated in the wide dissemination of mycoses of several species of scale insects in citrus orchards (Watson and Berger 1937, Fawcett 1948, McCoy 1985), especially the fungal genera *Aschersonia*, *Aegerita*, *Verticilium*, *Sphaerostilbe*, *Podonectria*, *Myriangium* and *Hirsutella* (Lord 2005). A complex of entomophthoralean fungi has been reported to infect other hemipteran insects (Barta and Cagan 2003). However, there are no data showing synergistic effects of pathogenic agents on mortality or spread of disease. Future research should focus on the virulence and infectivity of individual pathogens associated with *F. externa*. Equally as important is to determine the combined effects of multiple infections as these may accelerate the progress of disease and the spread of this epizootic. The fungi described in this paper associated with *F. externa* may be functioning as a natural regulatory factor in populations of this pest. Enhancing the impact of this complex of fungi through augmentative techniques could help to suppress *F. externa* populations in areas where the epizootic has not reached. This control could be achieved with low production costs and minimal environmental safety concerns.

Understanding the patterns and processes of the epizootic in *F. externa* populations in the Northeast will provide opportunities for the management of this pest in the future. Moreover, a better understanding of the possible synergistic actions among the fungi identified may elucidate the etiology of epizootics and fungal diseases in other insects. The entomopathogenic fungal species isolated from *F. externa* adults may prove to be
good candidates for the management of other hemipteran pests of hemlock forests, i.e., the hemlock woolly adelgid.
Literature Cited


CHAPTER 3

Colletotrichum acutatum f. sp. fiorinia infection of a scale insect, Fiorinia externa Ferris (Hemiptera: Diaspididae)

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Abstract: An epizootic has been reported in *Fiorinia externa* populations in New York, Connecticut, Pennsylvania and New Jersey. Infected insects have profuse sclerotia masses enclosing their bodies. The most commonly isolated microorganism from these infected *F. externa* was *Colletotrichum* sp. A morphological and molecular characterization of this fungus indicated that it is closely related to phytopathogenic *C. acutatum* isolates. Isolates of *Colletotrichum* sp. from *F. externa* in areas of the epizootic were genetically similar and were named *Colletotrichum acutatum* f. sp. *fiorinia* based on our findings. *In vitro* and *in planta* matings observed between isolates of *C. acutatum* f. sp. *fiorinia* could serve as a possible source of genetic variation may give rise to new biotypes with a propensity to infect insects. Only one other strain, *C. gloeosporioides* f. sp. *ortheziidae* has been reported to be entomopathogenic.

Key Words: *Fiorinia externa*, *Colletotrichum acutatum* f. sp. *fiorinia*, entomopathogenic fungi, epizootic

Introduction

The ascomycete *Colletotrichum* Corda (1831) is a widely occurring and intensively studied plant pathogen and research has focused on its effects on plants (Sutton, 1992). There is one reported case of a member of this genus, *C. gloeosporioides*, that causes epizootics on the scale *Orthezia praelonga* Douglas (1891) (Hemiptera: Coccoidea), a major pest of citrus in Brazil (Fig. 3.1S). It was first identified by Batista and Bezerra (1966) and morphologically confirmed by the Commonwealth Agriculture Bureau International, CABI (H.C. Evans, CABI report 1990). Biological control of *O. praelonga*, using *C. gloeosporioides*, has been conducted in Brazil (Viegas et al 1995,
Cesnik and Ferraz 2000) and scale mortality of 85-96% by 70 d after application has been obtained (Cesnik et al 1996).


Colletotrichum spp. employ different infection strategies in plants ranging from endophytic to hemibiotrophic and subcuticular intramural growth (Smith et al 1999). In many of the reported hosts, quiescent mesophyll intercellular mycelia rather than necrotrophic growth is observed (Wei et al 2004).

This paper presents the second report of a member of the genus Colletotrichum infecting an insect and the first report of infection of an insect by a species within Colletotrichum in North America. In 2002 an epizootic associated with a complex of fungi was observed in populations of elongate hemlock scale, Fiorinia externa Ferris (Hemiptera: Coccoidea), a pest of eastern hemlock [Tsuga canadensis (L.) Carrière] (McClure, 2002). The fungi associated with this epizootic appear to be of importance in the reduction of the F. externa population which together with the hemlock woolly...
adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae) are causing a significant decline in what has been one of the dominant components of New England’s forests for the last 8000 yr (Foster and Zebyrk 1993, Danoff-Burg and Bird 2002, Snyder et al 2002).

Black sclerotia masses covering 80-100% of *F. externa* populations were observed (Fig. 3.1T). Mortality of *F. externa* reached 36.8% (Marcelino et al. submitted).

Although the geographical origin of the epizootic is unknown presently it has been detected in 36 sites in New York, Connecticut, Pennsylvania and New Jersey.

The objective of this work was to morphologically, biologically and molecularly characterize the *Colletotrichum* sp. isolated from *F. externa* and to compare it to selected plant pathogenic strains in the same genus.

**Materials and Methods**

*Morphological plasticity.*—Morphological plasticity of 26 pure cultures of *Colletotrichum* sp. isolated from *F. externa* collected in Bayberry lane; Mohonk; Esopus and Ward Pound Ridge Reservation, in New York state, grown on potato dextrose agar media (PDA) maintained at 10, 15, 20 and 22 C was determined. The following fungal characteristics were visually monitored after 10 d: mycelium color, mycelium pigmentation, media pigmentation and presence and abundance of conidia masses. Monoconidial isolates and isolates derived from fungal suspensions in sterile distilled water were inoculated into 20 ml Potato Dextrose Agar (PDA) medium and cultured in Petri dishes. A stage-mounted DC3001 micromanipulator (World Precision Instruments, Inc., Sarasota, Florida, US) was used to obtain monoconidial isolates. The experiment was repeated three times for all isolates.
Sexual recombination.—Cross-fertile sexual recombination was attempted in vitro by testing all combinations of crosses between the following 20 d old cultures: *Colletotrichum* sp. isolates from the same and different localities of the epizootic; the single known entomopathogenic strain of *C. gloeosporioides* from Brazil (ARSEF4360); two plant pathogenic strains, ERL-1395 and ERL-1385, isolated from a golden delicious apple and a tulip tree (*Liriodendron tulipifera* L.) growing in the area of the epizootic (Table 3.1).

*In vitro* crosses were done on minimal salts medium (MSM), according to the protocol by Guerber and Correll (2001). Petri dishes were sealed with parafilm. Plates were incubated at room temperature (22 ± 0.5 C) with two constant cool white fluorescent lights placed 1.2 m above the plates and four additional lights 2.5 m away on the surrounding walls. Autoclaved birch wood (*Betula* sp.) toothpicks (Diamond Brand, Inc., Minneapolis), and excised 2 cm long strawberry stems sterilized with propylene oxide, were used as substrates to assess cross fertilization. Substrates were arranged in an N pattern. Mating plates were screened after 40 d to detect the presence of perithecia in the middle toothpick or stem, where the probability of selfing was reduced. The cross-fertilization experiment using toothpicks was repeated twice, whereas the one with strawberry stems was conducted once.

*In planta* sexual crossings were performed according to protocols from Cisar et al (1996), using 4.5 yr old hemlock seedlings [*Tsuga canadensis* (L.) Carrière], three month old bush snap bean (*Phaseolus vulgaris* L. var. Blue Lake 274) and three month old strawberry plants (*Fragaria x ananassa* Duchesne var. Honeoye) to determine if
Colletotrichum sp. (EHS\textsubscript{58}) obtained from \textit{F. externa} and \textit{Colletotrichum} sp. (ERL\textsubscript{1385}), isolated from a tulip tree could self and/or cross-fertilize. Strains of both fungi used in these experiments originated from an area of high incidence of the epizootic (Ward Pound Ridge Res., NY). Strain ERL\textsubscript{1385} was used because this \textit{Colletotrichum} sp. was repeatedly isolated from tulip trees and because molecular data confirmed that it was identical to the \textit{Colletotrichum} strain EHS\textsubscript{58} isolated from \textit{F. externa}. Two parallel sets of plants were set up. The first set included six plants per species while the second set included four. Plants were individually bruised with a sterile scalpel (four bruises/plant) and inoculated with 200 µl of 10\textsuperscript{6} suspension of either \textit{Colletotrichum} sp. EHS\textsubscript{58} or \textit{Colletotrichum} sp. ERL\textsubscript{1385}. Inoculated plants were held in a dew chamber (3.95 m high × 1.10 m wide × 1.10 m deep built with 2.5 cm diam PVC pipe and covered with plastic) for 24 h at 22 ± 1 C according to protocols of TeBeest (1988), and then placed in a greenhouse at 22 C for 8 d. After this period, the same bruises were inoculated with the alternate strain (ie EHS\textsubscript{58} followed by ERL\textsubscript{1385} or ERL\textsubscript{1385} followed by EHS\textsubscript{58} depending on the first strain initially inoculated). Re-inoculated plants were again held in the dew chamber for 24 h then moved to the greenhouse. For the first set of plants (six replicates), sample stems were excised weekly, whereas for the second set of plants (four replicates) the stems were excised at 3 and 6 wk. A 2 cm sample was excised from each plant and placed in MSM medium with penicillin (5ml/l) and streptomycin (12.5ml/l), and maintained in a growth chamber at a 8:16 h photoperiod (L:D) and 22 C. They were examined for the presence of the teleomorph stage (Glomerella) after 1 wk. The
experiment above was repeated using the bean plants, and inoculated with ERL1385 and another entomopathogenic strain, EHS48 from Bayberry Lane, NY.

*Measurement of fungal structures.*—Conidial spores were harvested from 14 d old monoconidial PDA cultures of 26 *Colletotrichum* sp. from the epizootic in the US and two *Colletotrichum* f. sp. ortheziidae from the epizootic in Brazil. Spores were stained with cotton blue lactophenol to enhance contrast. Appressoria were produced on 1 cm dm strawberry leaf discs. A drop of culture suspension was placed on individual leaf discs. Samples were incubated for 24 h at 22 °C in Petri dishes lined with moist filter papers. Appressoria were removed from leaves using the scotch tape print technique of Gouli et al. (2005), stained with cotton blue lactophenol to enhance visualization and avoid plant tissue interference. Conidia and appressoria were viewed using a 40x phase-contrast Trinocular BH2 Olympus compound microscope and photographed using a CCD digital camera (Pixelink, Vitana Corporation, Ottawa, Canada). Measurements of length and width were taken (N\textsubscript{conidia}=1751; N\textsubscript{appressoria}=1387), from which shape and elliptical form factors were calculated using the formulas below (Metamorph® software, Universal Imaging Corp., West Chester, Pennsylvania). Intact perithecia were removed from bean stems using a sterile insect pin and then placed in a glass slide, submerged in a droplet of distilled water and mounted with a cover slide and photographed at 40x in an Olympus BX51 photomicroscope connected to an AxioCam HC camera (Carl Zeiss Inc., Oberkochen, Germany). Ascospores extruded from perithecia were viewed in distilled water and photographed using the same procedures. Length, width, area and shape were calculated for both perithecia (N=40) and ascospores (N=119). In addition, the elliptical
form factor was calculated for the perithecia. The following values were calculated using Metamorph® software:

**Measurements:**

Spore length = \(\frac{1}{4}\left(\frac{\text{Perimeter} + \sqrt{\text{Perimeter}^2 - 16\text{Area}}}{2}\right)\)

Spore width = \(\frac{1}{4}\left(\frac{\text{Perimeter} - \sqrt{\text{Perimeter}^2 - 16\text{Area}}}{2}\right)\)

Shape Factor = \(\frac{4\pi\text{Area}}{\text{Perimeter}^2}\) (Circumference =1)

Elliptical Form Factor = \(\frac{\text{Length}}{\text{Breadth}}\) (Ratio of object’s breadth to its length)

P = Perimeter; A = Area

Appressorium length = Longest chord through the object

Appressorium width = Horizontal dimension of the object

Adapted from Metamorph® (2003)

**Statistical analysis.—** The SNK test (\(P=0.05\)) was used to identify whether there was a significant difference between the morphological parameters obtained for the isolates tested. Statistical analysis was performed using SAS® software (SAS Institute 1990).

**Phylogenetic analyses.—** Six Colletotrichum sp. pure strains obtained from F. externa were chosen for molecular analysis based on data for germination, growth rate and conidial production (Parker et al 2005). The rDNA from the following entomopathogenic and phytopathogenic strains was also sequenced: C. gloeosporiodes. f. sp. ortheziidae (ARSEF4360), from the USDA-ARS collection of entomopathogenic fungal cultures; C.
*gloeosporioides* f. sp. *ortheziidae* from EMBRAPA, Brazil (EMA26) and *C. acutatum*, ERL1379 and ERL1380 (TABLE 3.1).

The following genes were used to characterize selected isolates: D1/D2 region of the 28S ribosomal DNA, using primers NL1 and NL4 (O’Donnell 1992, 1993) commonly used for phylogenetic analysis at the genus level and above (Hillis and Dixon 1991); internal transcribed spacers ITS1 and ITS2, using primers ITS1 and ITS4 (White et al 1990) for within species differentiation (Afanador-Kafuri et al 2003); introns of the glyceraldehyde-3-phosphate dehydrogenase (GPDH), associated with the infection process of *Colletotrichum* (Wei et al 2004), with primers GDF1 and GDR1 (Templeton et al 1992); glutamine synthetase protein (GS), highly expressed during pathogenesis using primers GSF1 and GSR1 (Stephenson et al 1997), and newly constructed internal primers GLUintF1 (5′-AGCCGGAAGTCGGAGACATCCCG-3′) and GLUintR2 (5′-CGTTGCTGTTCTCCACGCAAT-3′). The β-Tubulin 2 protein encoding gene, used to distinguish fungi at deep phylogenetic levels (Thon and Royse 1999, Lubbe et al 2004), was amplified with primers TB5 and TB6 (Pannacione and Hanau 1990). The mating-type gene (MAT 1-2) from the High Mobility Box (HMG), used to study fungal biology (Turgeon 1998) was amplified with primers HMGacuF and HMGacuR (Du et al 2005).

A genetic characterization of representative *Colletotrichum* sp. isolates from the different regions of the epizootic, the Brazilian entomopathogenic *C. gloeosporioides* (ARSEF4360) and the phytopathogenic *C. acutatum* (ERL1379) was done using randomly amplified polymorphic DNA (RAPDs), with primers derived from repeated sequences GACA4.
DNA was extracted from 1 wk old cultures using the Power Soil™ DNA kit (Mo Bio Laboratories, Inc., Carlsbad, California), and the FastPrep™ FP120 machine (Thermo Savant, Holbrook, New York). Samples were shaken for 5 min at 5.5 m/s to break open fungal cell walls. The following modification was made to the Power Soil™ DNA kit; DNA was eluted using 100 µl of diluted elution buffer AE from Qiagen (1:15) containing 10 mM of Tris and 0.5 mM of EDTA, and concentrated down to 20 µl with a speed vacuum (Eppendorf Centrifuge 5415C, Vaudaux, Schönenbuch, Switzerland).

Polymerase Chain Reaction (PCR) was performed using Ready-To-Go PCR beads (Amersham Biosciences Inc., Piscataway, New Jersey). Genes were amplified using the following protocol: initial denaturation at 95 C for 2 min followed by 30 cycles of 95 C for 30 s (denaturation), 50 C for 30 s (annealing) and 72 C for 1 min (elongation) with the following changes in annealing temperature: ITS (52 C); GPDH (54 C); β-Tubulin 2 (65 C) and MAT 1-2 (55 C). RAPDs were amplified using Freeman et al. (1995) protocols. PCR products were purified using the Qiagen QIAquick® PCR purification kit (Valencia, CA) or Princeton Separations Centri Spin™ columns (Adelphia, New Jersey). DNA was stored at 4 C. PCR products were sequenced using BigDye v1 and BigDye v3 terminator cycle sequencing kit (Applied Biosystems, Foster City, California) using the following protocol: initial denaturation at 95 C for 3 min followed by 30 cycles of 95 C for 10 s (denaturation), 50 C for 5 s (annealing), and 60 C for 2 min (elongation).
Sequencing reactions were completed on a 3130xl Genetic Analyzer (Applied Biosystem, Foster City, California). Chromatograms were edited, and contiguous sequences were generated, using Sequencher™ (Gene Codes Corporation, Ann Arbor, Michigan). Sequences were analyzed with related sequences obtained from GenBank® (Templeton et al 1992, Dufresne 1997, Nirenberg et al 2002, Lubbe et al 2004, Du et al 2005, Talhinhas et al 2002, 2005) with the exception of the majority of GPDH and GS sequences which were not available in GenBank and were manually transcribed from Liu (2002). Introns were aligned individually with the aid of ClustalW (Chenna et al 2003) and retrieved with Jalview software (Clamp et al 2004). Exons were aligned separately based on their respective protein sequences using McClade (Maddison & Maddison 1992). Since, rDNA analyses have not been sufficient, so far, to resolve phylogenetic relationships in Colletotrichum spp. (Crouch et al 2005, Du et al 2005) the Colletotrichum spp. used as outgroups were based on their genetic distance to the strains used in the phylogenetic analysis. The following outgroup taxa were used: C. malvarum (Z18981) for the D1/D2 domain of the 28S gene; C. malvarum (949C3E55) and C. gloeosporioides (9E1589A5) for the GS gene; C. kahawae (AY376588) and C. gloeosporioides (AY376582); Glomerella cingulata (M93427) and G. cingulata (7BDBDAF86) for the GPDH gene; C. coccodes (AY3766528) for the ITS region and C. gloeosporioides (DQ002823) for the HMG at the MAT1-2 gene.

Phylogenetic trees were estimated using maximum parsimony as implemented in PAUP 4.0b10 (Swofford, 2002). One representative isolate was included in the analysis because the genomic sequences of Colletotrichum sp. from all the regions of the epizootic
were identical for all genes analyzed. Sequence gaps were treated as missing data. Bootstrap analysis was performed using 1000 Bootstrap replications with 30 random additions of taxa. Multiple equally parsimonious trees were combined into a single strict consensus tree. Only bootstrap values above 70 were included. Sequences used in this analysis were deposited in GenBank® (accession numbers included in TABLE 3.1). The sequence alignment for all the genes sequenced and respective phylogenetic trees, were also deposited in TreeBase databases (accession numbers to be assigned). Primers were excluded from published sequences and sequence alignments.

Results

Morphological plasticity.—The isolates used in this study showed a wide range of morphological variability often presenting several phenotypes as sectors within a colony (FIGS. 3.1J-N), for both single-spore and suspension derived isolates (TABLE 3.2).

Mycelium color ranged from gray or black when cultured on PDA at 10 and 15 C to pink or orange at 20 and 25 C. Multiple colors were at times present in the mycelia and the media (FIG. 3.1M). Chromogenic media pigmentation, typical of C. acutatum, was observed at 20 and 25 C in most of the isolates with gray and/or pink mycelium (FIG. 3.1O). Single spore isolates showed a stronger black color pigment in the media at 10 C than the suspension-derived isolates, which produced a gray color at 10 C. Only isolates EHS46, EHS50 and EHS51 produced orange mycelium consistently at all test temperatures. For the remaining isolates, gray mycelium pigment was observed at all temperatures. Aerial mycelium was seldom observed at all temperatures and isolates. Conidial masses (FIG. 3.1P) were not observed on single-spore cultures but did occur in cultures derived
from suspension at 20 C (TABLE 3.2). Statistical analysis was not performed on these data, as only the presence or absence of color pigment was recorded. In general, isolates grown at 10 C or 15 C (both single-spore or suspension-derived) produced gray mycelium, with sectors of other colors. In contrast, at 25 C, most of the isolates produced an equal combination of gray and pink mycelia with local sectors of other colors. Single-spore isolates were more uniform in color although color differences were common at 15 and 25 C.

**Sexual recombination.**—*In vitro* crossings were partially successful when using toothpicks as a substratum (TABLE 3.3). Cross-fertile recombination on toothpicks appeared to be incomplete in both repetitions of the experiment although tri-dimensional sterile structures were observed together with profuse conidial masses (FIG. 3.1R) especially on the diagonally-placed toothpicks (N). For EHS48 79% of the attempted crosses resulted in the production of these sterile structures. The phytopathogenic isolate (ERL1395) did not produce sterile structures in any of the crosses with the epizootic strains. The tulip tree isolate (ERL1385) produced sterile structures in 64% of the attempted crosses. The number of sterile structures was observed to be higher in crosses with isolates from different geographic origins. The cross-fertile bioassay using strawberry stems as a substratum produced profuse conidia masses (FIG. 3.1Q).

*In planta* cross-fertilizations with beans, strawberries and beans as substratum were successful only in beans. At 4 wk, and after 1 wk from excision from the plant, profuse numbers of perithecia were observed in self-fertile cross of EHS58 × EHS58. The cross-fertile ERL1385 × EHS58 produced perithecia after 5 wk *in planta* (FIG. 3.1A) and 1
wk after excised stems in petri-dishes (Fig. 3.1D). Perithecia were produced *in planta*, in two of the stem cuts in one of the experimental bean plants and *in vitro* in two bean stems cultured in MSM for 1 wk. Perithecia were not produced in a repeated experiment on beans. However, the perithecia produced in the bean stems in the first experiment were fertile, generating asci containing eight visible ascospores (Fig. 3.1B).

*Measurement of fungal structures.*— Wide ranges in the dimensions of the different propagules were measured. The mean conidial length of the 26 entomopathogen *Colletotrichum* sp. strains (Fig. 3.1I), was significantly smaller (5.61-8.57 × 2.73-4.22 μm) than the reported means for *C. acutatum* (Table 3.4). The ranges of means for Brazilian isolates of *C. gloeosporioides* f. sp. *ortheziidae*, EMA26 (Fig. 3.1G) and ARSEF4360 (Fig. 3.1H) were 10.35 × 2.50 μm and 11.88 × 2.37 μm, respectively. These ranges were significantly lower than those obtained for the *Colletotrichum* sp. isolates from sampled epizootic areas, with the exception of isolates EHS51, EHS52 and EHS56, which were within the range of widths for EMA26. Conidia of *C. gloeosporioides* f. sp. *ortheziidae* (EHS35) were more oblong shaped than the entomopathogenic *Colletotrichum* sp. from *F. externa*. In addition, the range of means for *C. gloeosporioides* f. sp. *ortheziidae* was also smaller than that reported for *C. gloeosporioides*. Only one strain of *Colletotrichum* sp., EHS41, was significantly different from all the other strains in terms of the spore area (Table 3.4).

The mean appressoria size (length × width) of the entomopathogenic *Colletotrichum* sp. (Fig. 3.1E), ranged from 6.35-7.85 × 5.58-6.85 μm and was similar to the reported range for *C. acutatum* (Table 3.5). The appressoria obtained from *C.
gloeosporioides f. sp. ortheziidae EMA_{26} (FIG. 3.1F), ranged from 8.11 × 6.90 µm and were also within the range for this species. *C. gloeosporioides*, ARSEF_{4360}, did not produce appressoria after numerous attempts on strawberry leaves, poinsettia leaves, *Euphorbia pulcherrima* and *Citrus* sp. leaves and fruit or mango, *Mangifera* sp., fruit). Although we found significant differences among isolates in the appressoria’s shape, they fell within the reported range in most cases. An average shape factor of 0.74-0.85 µm was calculated for *Colletotrichum* sp. (spherical=1) and 0.71 in EMA_{36}. The spore area for the 26 *Colletotrichum* strains and two *C. gloeosporioides* f. sp. ortheziidae strains were statistically identical (TABLE 3.5). Perithecia obtained from cross-fertile strains on plant stems after 7 d on MSM, averaged 115.39 × 108.83 µm, which was not within the reported range of *Glomerella acutata*. However, perithecia obtained from stems averaged 198.68 × 183.75 µm which is within the reported range for *G. acutata*. Perithecia retrieved *in planta* and from stems in MSM media were statistically different for length and width, shape and area (*P*<0.05) (TABLE 3.6).

No ovoid perithecia were found among specimens retrieved from plant stems, whereas in Petri dish cultures 21% of 26 perithecia were ovoid. The ratio of perithecium breadth to length (elliptical factor) was not statistically different for perithecia *in planta* and on MSM. The length was greater than the width, with a more marked difference in the perithecia from MSM. The area of the perithecia produced *in planta* (32211.60 µm²) was significantly different from those produced on MSM (8639.28 µm²). Ascospores discharged from all perithecia measured were smaller than those reported for *G. acutata* (TABLE 3.6). However, differences in area were found between ascospores from
perithecia in planta (FIG. 3.1C), 28.30 µm² and those produced on stems and cultured on MSM (39.29 µm²). These differences were also found in the shape factor (TABLE 3.7).

Molecular analysis.—A RAPDs analysis of seven representative isolates of Colletotrichum sp. from the epizootic and a single phytopathogenic C. acutatum isolated from blueberry (ERL1370) showed identical band patterns (FIG. 3.2). However, some genetic variation was observed, between the strains of Colletotrichum from the epizootic area in the northeastern US and the Colletotrichum sp. from the entomopathogenic C. gloeosporioides collected in Brazil (ARSEF4360).

We sequenced six genes from seven strains of Colletotrichum isolated from the F. externa epizootic and two C. gloeosporioides f. sp. ortheziidae strains from the epizootic in Brazil. These genes comprise a total of 3121 base pairs. Parsimony informative characters for the respective genes used in the analysis were as follows: GPDH (72.1%); HMG at the MAT1-2 (56.6%); GS (34.6%); β-Tubulin (26.2%); ITS (16.8%) and D1/D2 region of the 28S rDNA (3.4%). Colletotrichum and C. gloeosporioides nucleotide sequences obtained for the six genes where individually compared with related sequences from GenBank using BLAST. A subset of sequences with similarity at or above 90% were retrieved from GenBank and incorporated in the data set used for the phylogenetic analysis included herein. Colletotrichum isolated from F. externa and C. gloeosporioides isolated from O. praelonga were found to be most similar to known representative phytopathogenic C. acutatum species. For the ITS sequences, Colletotrichum from F. externa was identical to C. lupini (FIG. 3.3). The mean character difference for the respective genes used in the analysis between the two entomopathogenic Colletotrichum
spp., listed in decreasing order, is as follows: GPDH (8.4%); GS (5.9%); β-Tubulin2 (4.1%); HMG at MAT1-1 (3.7%); ITS (1.3%) and the D1/D2 region of the 28S rDNA (0.36%). We could not analyze a concatenated data set of all of the genes because sequences are not available for the same taxa in all genes. With the exception of the two ribosomal sequences, ITS and the 28S, the analysis from all other genes analyzed show strong support for the placement of the two entomopathogenic forms within a monophyletic *C. acutatum*, despite the different taxa used for each gene (Fig. 3.4, 3.5 and 3.6). The GPDH gene showed the greatest level of divergence between the *F. externa* derived *Colletotrichum* and *C. gloeosporioides* from *O. praelonga*, despite having sequenced 248 base pairs (Fig. 3.5). GPDH has the largest number of well supported branches, whereas 28S had the least.

**Discussion**

The work herein reports on the occurrence of *Colletotrichum acutatum* f. sp. *fiorinia* isolated from *F. externa* in 36 localities within the states of New York, Pennsylvania, Connecticut and New Jersey. Twenty-six entomopathogenic isolates of *Colletotrichum* sp. obtained from *F. externa*, originating from four different geographic localities of the epizootic were morphologically characterized to determine whether they were the same strain, and to compare them to known *Colletotrichum* species in order to assess their closest relative. Included in this analysis is the only other known *Colletotrichum* sp. to cause an epizootic in insects, *C. gloeosporioides* f. sp. *ortheziidae*, obtained from two distant geographic localities from its epizootic areas in Rio de Janeiro and São Paulo, Brazil.
Our morphological and molecular data indicated that the fungus isolated from *F. externa* in the epizootic belonged to the genus *Colletotrichum* and that it was identical to the species *C. acutatum*. *Colletotrichum* affects a wide variety of plants (Lenné 1992, Lardner et al 1999, Moriwaki et al 2002) with *C. acutatum* and *C. gloeosporioides* known to be the most cosmopolitan species. Reports of plant pathogens infecting insects are uncommon. Within the genus *Colletotrichum* there is only one other published case, that of *C. gloeosporioides* f. sp. *ortheziidae* infecting the scale *Orthezia praelonga* in Brazil (Cesnik and Ferraz, 2000). Our data indicate that *C. acutatum* f. sp. *fiorinia* is associated with the epizootic in *F. externa*. The phylogenetic analysis obtained from four of the six genes, ITS (Fig. 3.3), GS (Fig. 3.4), GPDH (Fig. 3.5) and β-tubulin2 suggests that the divergence in host utilization, from plant to insect, of both *C. acutatum* f. sp. *fiorinia* and *C. gloeosporioides* f. sp. *ortheziidae* are independent events. However, when using the HMG at the MAT1-2 gene both (Fig. 3.6) taxa form a monophyletic group, perhaps due to sampling error. Both *Colletotrichum* strains retrieved from *F. externa* and *O. praelonga* appear to be derived from *C. acutatum*.

With the exception of conidia and ascospore size, there is congruence between the two reliable morphological measurements and results obtained using molecular data. Thus we name this fungus isolated from *F. externa* in the northeastern epizootic region of the US is a *C. acutatum* f. sp. *fiorinia*.

We observed an array of phenotypes and sectors in cultures for *Colletotrichum* sp. recovered from *F. externa*. The highest level of variability in pigmentation occurred at 20 C and the lowest at 10 C. Although fungal phenotypic plasticity is a common
phenomenon *in vitro*, this strain was unusual in that we observed up to five different color pigments in both the mycelium and medium, in pure cultures. This level of variation indicates a high rate of morphological heterogeneity in this strain. In contrast, the two *C. gloeosporioides* f. sp. *ortheziidae* Brazilian strains tested did not display this plasticity in culture, presenting a consistent orange (ARSEF\textsubscript{4360}) and gray mycelia (EMA\textsubscript{26}) at both 15 C and 25 C.

Conidia of the 26 isolates of the entomopathogenic *Colletotrichum* sp., were smaller (5.61 to 8.57 µm) than the reported length for the *C. acutatum* species complex. The two entomopathogenic strains of *C. gloeosporioides* f. sp. *ortheziidae*, also produced smaller conidia, than the reported for *C. gloeosporioides* species complex. Appressoria length and width of both *Colletotrichum* sp. and *C. gloeosporioides* f. sp. *ortheziidae* were consistently within the lower range of that reported for *C. acutatum* and *C. gloeosporioides*. In both species appressoria were mainly ovoid.

Segregation of species based on morphological characters in the genus *Colletotrichum* has been based primarily on measurements of appressoria. Although we found that conidia size for both entomopathogenic strains from the US and Brazil are outside the reported range for other *Colletotrichum* strains, we do not believe this result alone is a reliable means at differentiating species segregation because the standard method for measuring spores is inconsistent. The method involves placing propagules, suspended in water, on a slide, where they can be oriented in different directions or can move due to brownian motion, hence biasing readings. In contrast, these problems are avoided when measuring appressoria using the scotch-tape print technique (Gouli et al...
Therefore it is not surprising that the appressoria measurements made for *Colletotrichum* sp. and *C. gloeosporioides* f. sp. ortheziidae fit within the range of *Colletotrichum* albeit in the lower range of the spectrum.

We detected sexual reproduction *in planta* using snap bean as a substratum, in crosses between *Colletotrichum* sp. strains from *F. externa* and a tulip tree from the epizootic area. Successful sexual reproduction was also detected *in vitro* after one week culturing of stems excised from beans, and cultured on MSM. However, reproduction was stalled when birch toothpicks were used as a substratum and no reproduction was observed with strawberry stems. Selfing was detected in a self-cross with the entomopathogenic *Colletotrichum* sp. (strain EHS$^{58}$ x EHS$^{58}$). Our data cannot differentiate as to whether the sexual cross obtained between the *Colletotrichum* sp. strain from *F. externa* and the *Colletotrichum* sp. from a tulip tree was homothallic or heterothallic since we obtained the perfect stage (*Glomerella*) with different and same strain crosses. However, these crosses do indicate that sexual reproduction can occur in this strain, possibly resulting in the adaptation to new hosts (Guerber and Correll 2001) and generation of new biotypes.

The crosses observed in beans were fertile. Perithecia, produced by the perfect stage of *Colletotrichum* (= *Glomerella*) generated asci containing eight ascospores. The mean range for the perithecia (length × width) retrieved after excised stems where placed in MSM were not within the reported range of *Glomerella acutata* (115.39 × 108.83 μm), whereas perithecia retrieved directly from stems *in planta* were within the reported range of *G. acutata* (198.68 × 183.43 μm). Size differences were also observed with perithecia
retrieved from excised stems after one week of culturing on MSM, presenting a mean area almost four times smaller than the area of the ones retrieved directly in planta (TABLE 3.6). Differences in the size of the ascospores produced in asci by the two types of perithecia were also found, with ascospores produced in perithecia directly retrieved from the stems being slightly smaller than the ones from perithecia in snap bean stems cultured in MSM. Incongruence between the size of perithecia and ascospores might result from differences in the environments in which the fruiting bodies were obtained. Perithecia values within the reported range were obtained from plants reared in a greenhouse with natural photoperiod, whereas smaller and out of range values where obtained from plant material in Petri dishes, in MSM media, in a growing chamber at a constant 8:16 h photoperiod (L:D) and sealed with parafilm. Like the previously reported conidial spore measurements, and for similar reasons discussed above, the ascospores of *Glomerella* sp. were not within the reported range for *G. acutata*.

Our molecular data indicate that a single population lineage of *Colletotrichum* from *F. externa*, henceforth referred as *Colletotrichum acutatum* f. sp. fiorinia, is present in the sampled epizootic area. A RAPDs analysis (FIG. 3.2) shows molecular homogeneity in *C. acutatum* f. sp. fiorinia strains sampled within the epizootic and a *C. acutatum* obtained from blueberry. However, differences were found between these apparently homologous strains and that obtained from *C. gloeosporioides* f. sp. ortheziidae.

A similar pattern was seen when six nuclear genes (ie the D1/D2 region of the 28S ribosomal DNA, ITS region, β-tubulin 2 gene, GPDH gene, GS gene and the High
Mobility Group of the mating-type gene, MAT1-2) were analyzed for these taxa. No differences were found between the *C. acutatum* f. sp. *fiorinia* strains collected within the area of the epizootic and *C. acutatum* from a blueberry. As with the RAPDs analysis, differences were found in the genes analyzed between *C. acutatum* f. sp. *fiorinia* and *C. acutatum* strains and *C. gloeosporioides* f. sp. *ortheziidae*.

A BLAST search of data in GenBank using sequences of the nuclear genes listed above, obtained from both *C. acutatum* f. sp. *fiorinia* and *C. gloeosporioides* f. sp. *ortheziidae*, retrieved records of *C. acutatum*, some of which were identical. In addition when using the ITS sequence we also retrieved another identical taxa, *C. lupini* (AJ301968).

Data collected indicates that *C. gloeosporioides* f. sp. *ortheziidae* appears to have attained specificity for *Orthezia praelonga* (Teixeira et al 2001, Jonsson and Genthner 1997, Castro et al 1998). This special form is currently being effectively used as a biological control agent in Brazil (Cesnik and Ferraz 2000) and under commercialization (R. Cesnik pers comm). It is possible that these two strains of *Colletotrichum* are at different stages of a host adaptation process. Data collected suggests that while *C. acutatum* f. sp. *fiorinia* still retains some capacity to invade plants endophytically (Marcelino et al. submitted), *C. gloeosporioides* f. sp. *ortheziidae* appears to have lost this capacity.

It has been suggested that *C. acutatum* may have a broader host range than what has been reported (Peres et al 2005). The cosmopolitan preference of this species for plants may pre-adapt it to infect radically different hosts. The means by which such wide
range of preference in hosts can be achieved by this pathogen are uncertain (Wei et al 2004). The ability to expand host range can result from genetic variation subsequent to sexual crossing. While we do not know whether this strain can produce an heterothallic cross we have induced an homothallic cross and generated the sexual stage, *Glomerella*, in our crosses. It is possible that this *C. acutatum* f. sp. *fiorinia* with its new propensity to infect insects rather than plants may have resulted from same sex mating. This type of reproductive strategy would produce meiotic clones, perhaps explaining the molecular homogeneity of the *C. acutatum* f. sp. *fiorinia* strains sampled within the area of the epizootic. A similar case of recombination via same-sex mating and subsequent expansion to new geographical niches has recently been reported for *Cryptococcus gattii* (Fraser et al 2005).

In most *Colletotrichum* spp. affecting plants the prevalent mode of reproduction is clonal, however, heterothallic intercompatibility has been reported (Roca et al 2004, Vaillancourt et al 2000, Crouch et al 2005).

The northeastern US forest area where the epizootic has occurred provides optimal conditions for the growth of *C. acutatum* f. sp. *fiorinia* which may have facilitated sexual crossing in this strain. Typical hemlock stands grow in riparian areas that help create a microclimate where relative humidity as a rule can reach 80% in the summer and attain 80 d/y of mist (Baldwin 1973, McGuire and Forman 1983).

The capacity of *Colletotrichum acutatum* f. sp. *fiorinia* to infect members of two kingdoms widens the host range for this species. The classification of members of this genus is in part determined by the identity of their hosts. The identification of a strain
which affects a wide variety of plants as well as an insect illustrates the difficulties inherent in using host identity for species segregation. This work indicates that multiple characters, including morphological and molecular, be used to classify member of this genus.

Acknowledgments

We thank Mr. Fred Little and Mr. Jeff Grosse for transcribing non-published genetic sequences in Liu, 2002. We also thank Dr. Felipe Soto for assistance with molecular analysis. This work was funded in part through a grant awarded by the Northeastern Area State and Private Forestry, USDA Forest Service (# 04-CA-11244225286) and is in partial fulfillment of requirements for the PhD degree of J.M. at the University of Vermont.
Literature Cited


**Fig. 3.1.** Perithecia *in planta* from cross fertile strains of *Colletotrichum* sp., ERL1383xEHS58 (A); intact ascus (B); free ascospores (C); Perithecia discharging ascospores (D); appressoria of *Colletotrichum* sp., strain EHS175, from epizootic site (E); appressorium of *C. gloeosporioides*, strain EMA26, from Brazilian epizootic (F); Conidia from *C. gloeosporioides*, EMA26 (G) and ARSEF4630 (H); *Colletotrichum* sp., strain EHS48, from epizootic area (I); morphological plasticity and overlapping phenotypes of *Colletotrichum* sp. strains from epizootic localities (J-M); Top (N) and reverse (O) of *Colletotrichum* sp. strain EHS58 in PDA at 25°C; Conidia produced by *Colletotrichum* sp. from epizootic site (P); Strawberry stem from cross fertile bioassay presenting profuse conidia masses (Q); Birch toothpick from cross fertile bioassay with conidia masses and sterile tri-dimensional structures (R); Adult *O. praelonga* infected with *C. gloeosporioides* f. sp. *orthezidae* (orange masses), Brazil (S); Adult *F. externa* with epizootic, US (T).

Bars: A = 200µm; B, C, E, F = 20µm; D = 60µm; G-I = 15µm, T = 5 mm.
FIG. 3.2. Random Amplified Polymorphic DNA (RAPDs) for *Colletotrichum* sp. strains from different geographic localities within the epizootic area. EHS$_{36}$ (A); EHS$_{41}$ (B); EHS$_{48}$ (C); EHS$_{51}$ (D); EHS$_{52}$ (E); EHS$_{58}$ (F); EHS$_{61}$ (G); from a phytopathogenic *C. acutatum* from blueberry, ERL$_{1379}$ (H); and from the Brazilian entomopathogenic *C. gloeosporioides* f. sp. *ortheziidae* ARSEF$_{4360}$ (I).
**Fig. 3.3.** Phylogenetic tree (Inferred MP tree) among *Colletotrichum* spp. and *Colletotrichum* sp. strains isolated from *Fiorinia externa*, for the ITS region. Consensus of three equally parsimonious trees. Numbers above nodes are bootstrap values based on 1000 iterations with 30 random additions each. Only branches with values above 70 are considered well supported.
**FIG. 3.4.** Phylogenetic tree (Inferred MP tree) among *Colletotrichum* spp. and *Colletotrichum* sp. strains isolated from *Fiorinia externa*, for the GS gene. Consensus of nine equally parsimonious trees. Numbers above nodes are bootstrap values based on 1000 iterations with 30 random additions each. Only branches with values above 70 are considered well supported.
**Fig. 3.5.** Phylogenetic tree (Inferred MP tree) among *Colletotrichum* spp. and *Colletotrichum* sp. strains isolated from *Fiorinia externa*, for the GPDH gene. Consensus of 350 equally parsimonious trees. Numbers above nodes are bootstrap values based on 1000 iterations with 30 random additions each. Only branches with values above 70 are considered well supported.
FIG. 3.6. Phylogenetic tree (Inferred MP tree) among *Colletotrichum* spp. and *Colletotrichum* sp. strains isolated from *Fioriia externa*, for the High Mobility Box at the MAT 1-2 gene. Consensus of two equally parsimonious trees. Numbers above nodes are bootstrap values based on 1000 iterations with 30 random additions each. Only branches with values above 70 are considered well supported.
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(*) Non-parametric ranking comprising overall strain performance based on germination rate, speed of growth and conidia production (Parker et al 2005).

(∆) Obtained from the USDA Entomopathogenic Fungal Collection, Ithaca, NY.

(∇) Obtained from EMBRAPA Colletotrichum gloeosporioides f. sp. ortheziidae, Jaguariúna, São Paulo, Brazil.

(†) Sequencing not performed.

(#) No amplification obtained with primers HMGacuF and HMGacuR.
### Table 3.2. Morphological plasticity in *Colletotrichum* sp. isolated in epizootic areas

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**TABLE 3.3. Cross-fertile bioassay in toothpick substratum**

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$+/- = \text{few stalled structures}; \quad + = \text{many stalled structures}; \quad - = \text{No structures}; \quad \text{Nt} = \text{Not tested}.$
**Table 3.4.** Conidia morphology of entomopathogenic *Colletotrichum* spp. at 22 C in PDA medium examined in water and stained with cotton blue

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<th>Strain of <em>Colletotrichum</em> sp. from US epizootic sites</th>
<th>Age (d)</th>
<th>N</th>
<th>Shape factor</th>
<th>Mean (µm)</th>
<th>Range</th>
<th>95% Confidence Interval</th>
<th>SD</th>
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<td>[6.25-7.01 x 2.84-3.15]</td>
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<td>19.71 D,E</td>
<td></td>
</tr>
<tr>
<td>EHS41</td>
<td>14</td>
<td>52</td>
<td>0.67 A,B</td>
<td>8.57 C x 3.95 A,B</td>
<td>[7.22-9.91 x 3.27-4.63]</td>
<td>4.82 x 2.44</td>
<td>43.78 A</td>
<td></td>
</tr>
<tr>
<td>EHS42</td>
<td>14</td>
<td>50</td>
<td>0.72 A,B</td>
<td>6.18 D,F x 3.52 D,C</td>
<td>[5.63-6.73 x 3.29-3.75]</td>
<td>1.94 x 0.81</td>
<td>22.30 C,E</td>
<td></td>
</tr>
<tr>
<td>EHS43</td>
<td>14</td>
<td>50</td>
<td>0.71 A,B</td>
<td>6.15 D,E,F x 3.38 C,F</td>
<td>[5.69-6.61 x 3.15-3.62]</td>
<td>1.62 x 0.82</td>
<td>20.84 D,E</td>
<td></td>
</tr>
<tr>
<td>EHS44</td>
<td>14</td>
<td>56</td>
<td>0.70 A,B</td>
<td>6.94 D,F x 3.72 D,C</td>
<td>[6.38-7.49 x 3.50-3.93]</td>
<td>2.05 x 0.80</td>
<td>25.59 C,E</td>
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</tr>
<tr>
<td>EHS45</td>
<td>14</td>
<td>99</td>
<td>0.68 A,B</td>
<td>6.56 D,F x 3.08 D,H</td>
<td>[6.09-7.02 x 2.93-3.23]</td>
<td>2.32 x 0.74</td>
<td>20.68 D,E</td>
<td></td>
</tr>
<tr>
<td>EHS46</td>
<td>14</td>
<td>81</td>
<td>0.73 A,B</td>
<td>7.15 D,E,F x 4.13 A</td>
<td>[6.69-7.62 x 3.98-4.28]</td>
<td>2.09 x 0.68</td>
<td>29.75 B,C</td>
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</tr>
<tr>
<td>EHS47</td>
<td>14</td>
<td>58</td>
<td>0.70 A,B</td>
<td>6.65 D,F x 3.49 C,E</td>
<td>[6.26-7.04 x 3.32-3.66]</td>
<td>1.48 x 0.65</td>
<td>23.13 C,E</td>
<td></td>
</tr>
<tr>
<td>EHS48</td>
<td>14</td>
<td>65</td>
<td>0.66 A,B</td>
<td>7.10 D,F x 3.27 C,F</td>
<td>[6.58-7.61 x 3.08-3.47]</td>
<td>2.06 x 0.77</td>
<td>22.65 C,E</td>
<td></td>
</tr>
<tr>
<td>EHS49</td>
<td>14</td>
<td>51</td>
<td>0.60 A,B</td>
<td>6.54 D,F x 3.33 C,F</td>
<td>[6.00-7.08 x 3.16-3.51]</td>
<td>1.93 x 0.62</td>
<td>21.39 D,E</td>
<td></td>
</tr>
<tr>
<td>EHS50</td>
<td>14</td>
<td>57</td>
<td>0.71 A,B</td>
<td>6.13 D,E,F x 3.06 D,H</td>
<td>[5.64-6.61 x 2.91-3.21]</td>
<td>1.82 x 0.57</td>
<td>19.29 D,E</td>
<td></td>
</tr>
<tr>
<td>EHS51</td>
<td>14</td>
<td>54</td>
<td>0.67 A,B</td>
<td>6.27 D,E,F x 2.80 G1</td>
<td>[5.69-6.85 x 2.63-2.98]</td>
<td>2.12 x 0.64</td>
<td>17.34 E</td>
<td></td>
</tr>
<tr>
<td>EHS52</td>
<td>14</td>
<td>99</td>
<td>0.66 A,B</td>
<td>6.26 D,E,F x 2.78 G1</td>
<td>[5.94-6.57 x 2.69-2.87]</td>
<td>1.68 x 0.47</td>
<td>17.26 E</td>
<td></td>
</tr>
<tr>
<td>EHS53</td>
<td>14</td>
<td>52</td>
<td>0.72 A,B</td>
<td>7.85 D,E,F x 4.22 A</td>
<td>[7.02-8.68 x 3.99-4.45]</td>
<td>2.97 x 0.81</td>
<td>33.45 B</td>
<td></td>
</tr>
<tr>
<td>EHS54</td>
<td>14</td>
<td>60</td>
<td>0.67 A,B</td>
<td>7.06 D,F x 3.32 C,F</td>
<td>[6.70-7.41 x 3.18-3.47]</td>
<td>1.36 x 0.55</td>
<td>23.31 C,E</td>
<td></td>
</tr>
<tr>
<td>EHS55</td>
<td>14</td>
<td>55</td>
<td>0.65 A,B</td>
<td>6.38 D,E,F x 2.73 H1</td>
<td>[5.89-6.87 x 2.58-2.88]</td>
<td>1.82 x 0.54</td>
<td>17.31 E</td>
<td></td>
</tr>
<tr>
<td>EHS56</td>
<td>14</td>
<td>64</td>
<td>0.71 A,B</td>
<td>6.44 D,E,F x 3.28 C,F</td>
<td>[5.97-6.91 x 3.15-3.42]</td>
<td>1.88 x 0.55</td>
<td>21.64 D,E</td>
<td></td>
</tr>
<tr>
<td>EHS57</td>
<td>14</td>
<td>58</td>
<td>0.69 A,B</td>
<td>6.64 D,E,F x 3.24 C,G</td>
<td>[6.00-7.28 x 3.05-3.43]</td>
<td>2.44 x 0.72</td>
<td>21.40 D,E</td>
<td></td>
</tr>
<tr>
<td>EHS58</td>
<td>14</td>
<td>51</td>
<td>0.69 A,B</td>
<td>6.75 D,E,F x 3.57 D,C</td>
<td>[6.08-7.43 x 3.31-3.84]</td>
<td>2.40 x 0.94</td>
<td>22.97 C,E</td>
<td></td>
</tr>
<tr>
<td>EHS59</td>
<td>14</td>
<td>79</td>
<td>0.71 A,B</td>
<td>6.01 D,E,F x 3.29 C,F</td>
<td>[5.64-6.37 x 3.13-3.45]</td>
<td>1.61 x 0.71</td>
<td>19.56 D,E</td>
<td></td>
</tr>
<tr>
<td>EHS60</td>
<td>14</td>
<td>75</td>
<td>0.71 A,B</td>
<td>5.96 D,E,F x 3.14 D,H</td>
<td>[5.55-6.36 x 2.99-3.29]</td>
<td>1.76 x 0.63</td>
<td>18.55 D,E</td>
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</tr>
<tr>
<td>EHS61</td>
<td>14</td>
<td>54</td>
<td>0.67 A,B</td>
<td>6.80 D,E,F x 3.10 D,H</td>
<td>[6.18-7.41 x 2.88-3.31]</td>
<td>2.24 x 0.79</td>
<td>20.45 D,E</td>
<td></td>
</tr>
</tbody>
</table>

(Continue)
TABLE 3.4 (Continued). Conidia morphology of entomopathogenic *Colletotrichum* spp. at 22 C in PDA medium examined in water and stained with cotton blue

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age (d)</th>
<th>N</th>
<th>Shape factor</th>
<th>Conidial length × width (µm)</th>
<th>Mean (µm)</th>
<th>Range (µm)</th>
<th>95% Confidence Interval</th>
<th>SD</th>
<th>Area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Range</strong></td>
<td></td>
<td>0.65-0.73</td>
<td>0.69</td>
<td>5.61-8.57 × 2.73-4.22</td>
<td>[ 4.78-9.91 × 2.55-4.63 ]</td>
<td>1.35-4.82 × 0.47-2.44</td>
<td>[ 6.51-6.73 × 3.26-3.35 ]</td>
<td>2.22 × 0.91</td>
<td>17.26-43.78</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td>6.62 × 3.31</td>
<td></td>
<td>6.25-8.57 × 2.73-4.22</td>
<td>[ 4.78-9.91 × 2.55-4.63 ]</td>
<td>1.35-4.82 × 0.47-2.44</td>
<td>[ 6.51-6.73 × 3.26-3.35 ]</td>
<td>2.22 × 0.91</td>
<td>22.42</td>
</tr>
</tbody>
</table>

*Colletotrichum gloeosporioides* from Brazilian epizootic sites

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age</th>
<th>N</th>
<th>Shape factor</th>
<th>Conidial length × width (µm)</th>
<th>Mean (µm)</th>
<th>Range (µm)</th>
<th>95% Confidence Interval</th>
<th>SD</th>
<th>Area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMA26</td>
<td>14</td>
<td>99</td>
<td>0.49 ^c</td>
<td>10.35 ^b × 2.50 ^l</td>
<td>[ 9.80-10.92 × 2.39-2.62 ]</td>
<td>2.76 × 0.55</td>
<td>26.17 ^c ^e</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARSEF230</td>
<td>14</td>
<td>73</td>
<td>0.44 ^c</td>
<td>11.88 ^a × 2.37 ^i</td>
<td>[11.11-12.66 × 2.23-2.52 ]</td>
<td>3.33 × 0.62</td>
<td>27.31 ^c ^d</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values with different letters are statistically different according to the Student-Newman Keuls (SNK) post-hoc test performed using SAS®.

* Reported range of means (length x width) for *C. acutatum* in PDA medium at 21-25 C; examined in water = 13.2-15.9 × 4.4-4.9 µm.

* *C. gloeosporioides* (self-sterile strains) = 15.0 - 16.6 × 5.3-5.5 µm; *C. gloeosporioides* (self-fertile strains) = 14.8 - 16.3 × 5.4-5.5 µm.

(Du et al 2005).

* Reported range of means (length x width) for *C. acutatum* in PDA medium at 22 C, examined in water = 9.3-16.9 × 3.1-5.4 µm.

* *C. gloeosporioides* = 14.7-18.8 × 5.0-6.2 µm.

(Guerber and Correll 2001).
TABLE 3.5. Appressoria morphology of entomopathogenic *Colletotrichum* sp. strains growing in strawberry leaves at 22 C examined in water and stained with cotton blue

<table>
<thead>
<tr>
<th>Straina</th>
<th>N</th>
<th>Shape factor</th>
<th>Perimeter</th>
<th>Mean (µm)</th>
<th>Range 95% Confidence Interval</th>
<th>SD</th>
<th>Area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Appressoria length × width (µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHS35</td>
<td>53</td>
<td>0.80 A,E</td>
<td>23.20 A</td>
<td>7.80 A,C × 6.75 A,C</td>
<td>[ 7.47-8.14 × 6.49-7.01 ]</td>
<td>1.19 × 0.94</td>
<td>34.31 A</td>
</tr>
<tr>
<td>EHS36</td>
<td>48</td>
<td>0.83 A,B</td>
<td>19.67 D,E</td>
<td>6.82 E-G × 5.91 D-H</td>
<td>[ 6.63-7.02 × 5.66-6.16 ]</td>
<td>0.67 × 0.85</td>
<td>25.68 F-H</td>
</tr>
<tr>
<td>EHS37</td>
<td>48</td>
<td>0.77 B-E</td>
<td>19.87 E</td>
<td>6.35 J × 5.58 H</td>
<td>[ 6.18-6.52 × 5.40-5.76 ]</td>
<td>0.57 × 0.65</td>
<td>22.46 J</td>
</tr>
<tr>
<td>EHS39</td>
<td>49</td>
<td>0.78 B-F</td>
<td>20.56 C-E</td>
<td>7.03 D1 × 6.11 C-H</td>
<td>[ 6.80-7.26 × 5.79-6.43 ]</td>
<td>0.81 × 1.11</td>
<td>25.66 F-H</td>
</tr>
<tr>
<td>EHS40</td>
<td>38</td>
<td>0.80 A-D</td>
<td>20.52 C-E</td>
<td>7.13 D1 × 6.16 B-H</td>
<td>[ 6.88-7.37 × 5.88-6.44 ]</td>
<td>0.74 × 0.85</td>
<td>27.84 D-H</td>
</tr>
<tr>
<td>EHS41</td>
<td>63</td>
<td>0.81 A-D</td>
<td>19.05 E</td>
<td>6.73 G1 × 5.62 G1</td>
<td>[ 6.54-6.92 × 5.41-5.84 ]</td>
<td>0.75 × 0.85</td>
<td>23.49 L, J</td>
</tr>
<tr>
<td>EHS42</td>
<td>46</td>
<td>0.79 B-F</td>
<td>19.58 D,E</td>
<td>6.68 B1 × 5.87 E-H</td>
<td>[ 6.47-6.89 × 5.64-6.09 ]</td>
<td>0.69 × 0.75</td>
<td>24.22 H, J</td>
</tr>
<tr>
<td>EHS43</td>
<td>58</td>
<td>0.81 A-D</td>
<td>19.59 D,E</td>
<td>6.76 G1 × 5.91 D1</td>
<td>[ 6.55-6.97 × 5.69-6.13 ]</td>
<td>0.70 × 0.84</td>
<td>24.78 G, J</td>
</tr>
<tr>
<td>EHS44</td>
<td>54</td>
<td>0.77 B-F</td>
<td>20.70 C-E</td>
<td>7.31 B-H × 6.29 A-G</td>
<td>[ 6.99-7.63 × 5.90-6.67 ]</td>
<td>1.16 × 1.40</td>
<td>26.20 F-H</td>
</tr>
<tr>
<td>EHS45</td>
<td>55</td>
<td>0.78 B-F</td>
<td>19.68 D,E</td>
<td>6.61 D1 × 5.69 F-H</td>
<td>[ 6.40-6.61 × 5.51-5.86 ]</td>
<td>0.74 × 0.64</td>
<td>24.22 H, J</td>
</tr>
<tr>
<td>EHS46</td>
<td>35</td>
<td>0.82 A-C</td>
<td>22.52 A,B</td>
<td>7.85 A,B × 6.85 A,B</td>
<td>[ 7.52-8.17 × 6.47-7.23 ]</td>
<td>0.95 × 1.10</td>
<td>33.32 A,B</td>
</tr>
<tr>
<td>EHS47</td>
<td>61</td>
<td>0.85 A</td>
<td>21.22 B-D</td>
<td>7.06 D1 × 6.20 B-H</td>
<td>[ 6.92-7.21 × 6.86-6.33 ]</td>
<td>0.56 × 0.51</td>
<td>30.50 B-D</td>
</tr>
<tr>
<td>EHS49</td>
<td>42</td>
<td>0.79 B-F</td>
<td>21.03 B-D</td>
<td>7.29 B-H × 6.18 B-H</td>
<td>[ 7.02-7.56 × 5.90-6.46 ]</td>
<td>0.85 × 0.90</td>
<td>28.06 D-G</td>
</tr>
<tr>
<td>EHS50</td>
<td>66</td>
<td>0.77 B-F</td>
<td>22.59 A,B</td>
<td>7.61 A-D × 6.53 A-E</td>
<td>[ 7.36-8.76 × 6.29-6.77 ]</td>
<td>1.01 × 0.98</td>
<td>31.51 B,C</td>
</tr>
<tr>
<td>EHS51</td>
<td>78</td>
<td>0.81 A-D</td>
<td>20.58 C-E</td>
<td>7.20 C-D × 6.03 D-H</td>
<td>[ 6.99-7.42 × 5.81-6.25 ]</td>
<td>0.94 × 0.96</td>
<td>27.44 D-H</td>
</tr>
<tr>
<td>EHS52</td>
<td>49</td>
<td>0.79 B-F</td>
<td>21.76 A-C</td>
<td>7.53 B-D × 6.50 A-E</td>
<td>[ 7.28-7.77 × 6.19-6.81 ]</td>
<td>0.85 × 1.07</td>
<td>30.08 C-E</td>
</tr>
<tr>
<td>EHS53</td>
<td>53</td>
<td>0.83 A,B</td>
<td>19.79 D,E</td>
<td>7.06 D1 × 5.96 D-H</td>
<td>[ 6.87-7.25 × 5.76-6.16 ]</td>
<td>0.68 × 0.73</td>
<td>26.06 F-H</td>
</tr>
<tr>
<td>EHS55</td>
<td>48</td>
<td>0.78 B-F</td>
<td>21.07 B-D</td>
<td>7.17 C-D × 6.34 A-F</td>
<td>[ 6.88-7.45 × 6.11-6.57 ]</td>
<td>0.97 × 0.80</td>
<td>27.77 D-H</td>
</tr>
<tr>
<td>EHS56</td>
<td>53</td>
<td>0.81 A-D</td>
<td>21.10 B-D</td>
<td>7.42 B-D × 6.33 A-G</td>
<td>[ 7.20-7.63 × 6.05-6.60 ]</td>
<td>0.78 × 0.99</td>
<td>28.78 C-F</td>
</tr>
<tr>
<td>EHS57</td>
<td>62</td>
<td>0.77 C-F</td>
<td>21.75 A-C</td>
<td>7.37 B-G × 6.26 A-B</td>
<td>[ 7.18-7.55 × 6.04-6.48 ]</td>
<td>0.72 × 0.87</td>
<td>28.83 C-F</td>
</tr>
<tr>
<td>EHS58</td>
<td>50</td>
<td>0.77 B-F</td>
<td>21.29 B-D</td>
<td>7.18 C-D × 6.54 A-E</td>
<td>[ 6.90-7.46 × 6.22-6.86 ]</td>
<td>0.99 × 1.12</td>
<td>27.78 D-H</td>
</tr>
<tr>
<td>EHS59</td>
<td>52</td>
<td>0.75 E-F</td>
<td>21.26 B-D</td>
<td>7.19 C-D × 6.02 D-H</td>
<td>[ 6.87-7.51 × 5.66-6.39 ]</td>
<td>1.14 × 1.30</td>
<td>26.82 E, J</td>
</tr>
<tr>
<td>EHS61</td>
<td>11</td>
<td>0.76 D-F</td>
<td>21.36 B-D</td>
<td>7.47 B-D × 6.39 A-F</td>
<td>[ 6.84-8.09 × 5.53-7.25 ]</td>
<td>0.92 × 1.27</td>
<td>28.07 D-G</td>
</tr>
</tbody>
</table>

(Continue)
### TABLE 3.5 (Continued). Appressoria morphology of entomopathogenic *Colletotrichum* sp. strains growing in strawberry leaves at 22 °C examined in water and stained with cotton blue

<table>
<thead>
<tr>
<th>Strain</th>
<th>N</th>
<th>Shape factor</th>
<th>Perimeter</th>
<th>Mean (µm)</th>
<th>Range 95% Confidence Interval</th>
<th>SD</th>
<th>Area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHS63</td>
<td>72</td>
<td>0.79 B-F</td>
<td>22.22 A-C</td>
<td>7.50 B-D × 6.62 A-D</td>
<td>[ 7.31-7.69 × 6.42-6.82 ]</td>
<td>0.80 × 0.85</td>
<td>31.45 B,C</td>
</tr>
<tr>
<td>EHS66</td>
<td>43</td>
<td>0.79 B-F</td>
<td>21.77 A-C</td>
<td>7.58 A-D × 6.31 A-G</td>
<td>[ 7.34-7.83 × 6.03-6.59 ]</td>
<td>0.79 × 0.90</td>
<td>29.95 C-E</td>
</tr>
</tbody>
</table>

Range

Mean

Colletotrichium gloeosporioides from Brazilian epizootic sites

<table>
<thead>
<tr>
<th>Strain</th>
<th>N</th>
<th>Shape factor</th>
<th>Perimeter</th>
<th>Mean (µm)</th>
<th>Range 95% Confidence Interval</th>
<th>SD</th>
<th>Area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMA26</td>
<td>41</td>
<td>0.71 G</td>
<td>23.03 A</td>
<td>8.11 A × 6.90 A</td>
<td>[ 7.60-8.62 × 6.46-7.35 ]</td>
<td>1.61 × 1.41</td>
<td>30.38 B-D</td>
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<tr>
<td>ARSEF456</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

- Measurements were made after 24 h.
- Values with different letters are statistically different according to the Student-Newman Keuls (SNK) post-hoc test performed using SAS®
- Reported range for *C. acutatum* appressoria (length x width) produced in deionized H₂O, in a moist chamber overnight = 5.8-10.3 × 4.6-9.5 µm;

*C. gloeosporioides* = 5.7-8.8 × 4.2-6.6 µm.

(Du et al. 2005).
**Table 2.6.** Perithecia of *Glomerella* sp. on snap bean stems (2 cm) at 22 C from cross-fertile ERL1385 × EHS88

<table>
<thead>
<tr>
<th>Samples(\d)</th>
<th>N</th>
<th>Elliptical Factor</th>
<th>Shape factor</th>
<th>Form (%)</th>
<th>Perithecia length × width (µm)</th>
<th>Mean width (µm)</th>
<th>SD</th>
<th>Area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep. 2(\d)</td>
<td>26</td>
<td>1.20 A</td>
<td>0.83 A</td>
<td>46 A</td>
<td>115.39 A × 108.83 A</td>
<td>29.62 × 29.75</td>
<td>8639.28 A</td>
<td></td>
</tr>
<tr>
<td>Rep. 2-a(\d)</td>
<td>14</td>
<td>1.09 A</td>
<td>0.62 B</td>
<td>64 B</td>
<td>198.68 B × 183.75 B</td>
<td>98.36 × 92.06</td>
<td>32211.60 B</td>
<td></td>
</tr>
<tr>
<td>Mean(\d)</td>
<td>1.16</td>
<td>0.75</td>
<td>146.08 × 136.43</td>
<td>74.85 × 69.78</td>
<td>17323.82</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Measurements for self-fertile sample were not estimated due to copious saprophytic growth in the sample.

\(\d\) Perithecia produced in bean stem retrieved from the plant after three wk inoculation and seven d on MSM;

\(\d\) Perithecia retrieved directly from the plant stem;

\(\d\) Values with different letters are statistically different according to the Student-Newman Keuls (SNK) post-hoc test performed using SAS®;

\(\d\) Reported range of mean width for *G. acutata* retrieved from crosses in birch toothpicks on minimal salts medium (MSM), at 22 C, and after 26-41 d, under constant fluorescent light, and examined in water = 156-203 µm (Guerber and Correll 2001);

\(\d\) Perithecium form: A= Ampulliform; G= Globose; O=Ovoid.
**TABLE 3.7.** Ascospores of *Glomerella* sp. on snap bean stems (2 cm) at 22°C and under natural light

<table>
<thead>
<tr>
<th>Samples</th>
<th>N</th>
<th>Shape factor</th>
<th>Mean (µm)</th>
<th>Range 95% Confidence Interval</th>
<th>SD</th>
<th>Area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep.2</td>
<td>64</td>
<td>0.47 A</td>
<td>9.12 A × 3.16 A</td>
<td>[ 8.78-9.45 × 3.05-3.28 ]</td>
<td>1.34 × 0.46</td>
<td>39.29 A</td>
</tr>
<tr>
<td>Rep.2-a</td>
<td>55</td>
<td>0.57 B</td>
<td>9.17 A × 3.02 A</td>
<td>[ 8.84-9.50 × 2.91-3.13 ]</td>
<td>1.22 × 0.40</td>
<td>28.30 B</td>
</tr>
<tr>
<td>Range</td>
<td>0.47-0.57</td>
<td>9.12-9.17 × 3.02-3.16</td>
<td>[ 8.78-9.50 × 2.91-3.13 ]</td>
<td>1.34-1.22 × 0.40-0.46</td>
<td>28.30-39.29</td>
<td></td>
</tr>
</tbody>
</table>

Measurements for self-fertile sample were not estimated due to copious saprophytic growth in the sample.

*Values with different letters are statistically different according to the Student-Newman Keuls (SNK) post-hoc test performed using SAS®; reported range of means (length x width) for *G. acutata* retrieved from crosses in birch toothpicks; on MSM; after 26-33 d; at 20°C under constant fluorescent light and examined in water = 12.2-17.7 × 5.0-6.5 µm (Guerber and Correll 2001).
CHAPTER 4

Host Plant Associations with an Entomopathogenic Strain of Colletotrichum acutatum from Elongate Hemlock Scale

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Abstract

A fungal epizootic has been detected in populations of Fiorinia externa in hemlock forests of several northeastern states. Colletotrichum acutatum, a well-known plant pathogen, was the most commonly recovered fungus from infected scales. This is the second report of a Colletotrichum sp. infecting scale insects. In Brazil C. gloeosporioides f. sp. ortheziidae recovered from Orthezia praelonga is under development as a biopesticide for citrus production. The potential of developing the Fiorinia strain for management of F. externa is being evaluated. This fungus was also detected growing endophytically in 28 species of plants within the epizootic areas. Using molecular methods, it was determined that the Colletotrichum strains recovered from scales (C. acutatum f. sp. fiorinia) and plants were identical for the High Mobility Box at the MAT1-2, mating type gene. Results from plant bioassays confirmed that this entomopathogenic Fiorinia strain exhibits the capacity to grow endophytically in several of the plants tested without causing external symptoms or signs of infection. In addition, this strain causes mild symptoms of infection in strawberry plants. The implications of these findings for the use of this fungus as a biological control agent are discussed.

Keywords: Fiorinia externa, Colletotrichum sp., epizootic, elongate hemlock scale, host range
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Introduction

The eastern hemlock, *Tsuga canadensis* (L.) Carrière, a common species in forests in the northeastern United States, is showing evidence of significant decline in some areas. One causal agent associated with this decline is *Fiorinia externa* Ferris (Hemiptera: Diaspididae), elongate hemlock scale, an exotic invasive from Japan accidentally introduced in the early 1900s (Sasscer 1912, Lambdin et al. 2005). Chemical insecticides and classical biological control methods have proven impractical and provided inconsistent efficacy (Smith and Lewis 2005). Considering the value of eastern hemlock to forest biodiversity, effective methods to manage *F. externa* are needed.

In 2002 an epizootic was reported within a population of *F. externa* in the Mianus River Gorge Preserve in Bedford, NY and attributed to unidentified fungi which produced sclerotia that often completely covered the insect (McClure 2002). Several additional epizootic sites have been identified in 36 different geographical localities in New York, Pennsylvania, Connecticut and New Jersey. The most prevalent fungus isolated from these epizootics has been morphologically and molecularly identified as *Colletotrichum acutatum* f. sp. *fiorinia*, the *Fiorinia* strain (unpublished data) and has shown the ability to be easily cultured *in vitro* (Parker et al. 2005).

Though *C. acutatum* is more commonly known as a phytopathogen (Bailey and Jeger 1992, Prusky et al. 2000), one report was found of *C. gloeosporioides* f. sp. *ortheziidae* causing epizootics in the scale *Orthezia praelonga* Douglas 1891 (Hemiptera: Ortheziidae), a major pest of *Citrus* spp. in Brazil. Research on the biological control of this pest using the *Orthezia* strain has been conducted in Brazil for >13 yr and this strain
is under commercial development (Cesnik and Ferraz 2000, R. Cesnik, pers. comm. 2006). Infection of *F. externa* by the *Fiorinia* strain represents the second reported case of a member of this genus infecting a scale insect.

The distribution and infective capacity of the *Fiorinia* strain must be assessed to evaluate the biological control potential of this organism. An assessment of its natural occurrence in plants in the hemlock forest ecosystem and its phytopathogenicity to several horticultural crops were made. This information is critical before further development of the *Fiorinia* strain for use against *F. externa* can be considered.

**Material and Methods**

**Plant bioassays**

*Isolates.* Several entomopathogenic and phytopathogenic fungi were tested to determine their ability to infect horticultural plants and eastern hemlock seedlings (Table 4.1). The following strains of *C. acutatum* were tested: five entomopathogenic strains of *C. acutatum* f. sp. *fiorinia* (hereafter called *Fiorinia* strains) obtained from pure culture lines isolated from *F. externa* in sites where the epizootic occurs; and two phytopathogenic *C. acutatum* strains, one from a blueberry fruit (ERL1379) and one from a tomato fruit (ERL1380). To compare the phytopathogenicity of the *Fiorinia* strains with a recognized entomopathogen, one strain of *Lecanicillium lecanii* (Zimmerman) Gams & Zare [= *Verticillium lecanii* (Zimm.) Viégas] (EHS132) isolated from *F. externa* was included in the bioassays. In the 2006 bioassays, the entomopathogenic *C. gloeosporioides* f. sp. *ortheziidae* (ARSEF4360) from Brazil was also included. Isolates were grown in potato dextrose agar (PDA) (39 g/l) supplemented with penicillin (5 ml/l)
and streptomycin (12.5 ml/l) for 10-12 d before being harvested with sterile Pasteur pipettes to obtain suspensions of the isolates, in sterile distilled water (SDW), for subsequent calibration of conidial concentrations.

**Table 4.1. Fungal isolates used for the horticultural plant and hemlock phenological bioassays**

<table>
<thead>
<tr>
<th>Fungus type</th>
<th>Species</th>
<th>Code</th>
<th>Host</th>
<th>Geographic origin</th>
<th>Year of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entomopathogenic fungi</td>
<td><em>Colletotrichum</em> f. sp. <em>Fiorinia</em> (F. <em>fiorinia</em> strain)</td>
<td>EHS41</td>
<td><em>Fiorinia</em> externa</td>
<td>Mohonk, NY</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td><em>Fiorinia</em> strain</td>
<td>EHS48</td>
<td><em>F. externa</em></td>
<td>Bayberry Lane, NY</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td><em>Fiorinia</em> strain</td>
<td>EHS51</td>
<td><em>F. externa</em></td>
<td>Esopus, NY</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td><em>Fiorinia</em> strain</td>
<td>EHS58</td>
<td><em>F. externa</em></td>
<td>Ward Pound Ridge Reservation, NY</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td><em>Fiorinia</em> strain</td>
<td>EHS61</td>
<td><em>F. externa</em></td>
<td>Ward Pound Ridge Reservation, NY</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td><em>Lecanicilium lecanii</em></td>
<td>EHS132</td>
<td><em>F. externa</em></td>
<td>South Salem, NY</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td><em>C. gloeosporiodes</em> f. sp. ortheziidae</td>
<td>ARSEF4360</td>
<td><em>Orthezia</em> praelonga</td>
<td>Jaguariuna, Sao Paulo, Brazil</td>
<td>1994</td>
</tr>
<tr>
<td>Phytopathogenic fungi</td>
<td><em>C. acutatum</em></td>
<td>ERL-1379</td>
<td>Blueberry fruit</td>
<td>NJ</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td><em>C. acutatum</em></td>
<td>ERL-1380</td>
<td>Tomato fruit</td>
<td>Burlington, VT</td>
<td>2005</td>
</tr>
</tbody>
</table>

**Plants.** The virulence of the above fungal isolates was tested on the following horticultural crops: pepper (*Capsicum annuum* var. New Ace, Solanaceae), tomato
(Solanum lycopersicum var. Patio, Solanaceae); common bush snap bean (Phaseolus vulgaris var. Blue Lake 274, Fabaceae); strawberry (Fragaria x ananassa var. Honeoye, Rosaceae) and barley (Hordeum vulgare, Gramineae). These plant species were selected for testing because they are reported to be highly susceptible to C. acutatum (Ivey et al. 2004, Dillard and Cobb 1998, Tu 1992, Horowitz et al. 2004, Martin and Skoropad 1978). All plants were grown in a greenhouse from seed in 23-cm diam pots containing Metro-Mix 360 potting medium (Sungro Horticulture, Vancouver, Canada). Four wk old plants were used for these bioassays. In addition to the horticultural plants, 4 yr old eastern hemlock seedlings (Western Maine Nurseries, Fryeberg, ME) were also tested. Only plants with no external signs of fungal infection or disease were selected for testing.

Each plant was sprayed individually to runoff with 30 ml of a suspension of 10⁶ conidia/ml SDW containing 0.02% Silwet L-77 as a wetting agent (Loveland Industries, Inc., Greeley, CO). Suspensions were calibrated to the correct conidial concentration per ml SDW with an Improved Neubauer haemacytometer (Propper®) according to the protocol of Goettel and Inglis (1997). Suspensions were applied with a hand-held air-brush sprayer (Badger Air-Brush Co., Franklin Park, IL) operating at 1.75 kg/cm² in a sterile fume hood. Controls were treated with SDW containing 0.02% Silwet L-77. After treatment, plants were incubated in a dew chamber (3.95 m high × 1.10 m wide × 1.10 m deep built with 2.5 cm diam PVC pipe and covered with plastic) for 24 h at 22 ± 1°C according to protocols of TeBeest (1988), then transferred to a naturally illuminated greenhouse with adjusted temperature (22 ± 0.5°C), and arranged on benches in a
completely randomized block design. Each treatment was replicated four times per plant species, and the complete bioassay was run once in 2005 and 2006.

Koch’s postulates were performed 1 mo after treatment. Four to six leaf samples (approx. 2 sq. cm) were excised from each plant and surface sterilized by immersion in 75% ethanol + 0.02% Silwet L-77 for 20 s (Cowles et al. 2000), followed by 5 s in SDW, 45 s in 2.5% sodium hypochlorite (NaOCl) and finally rinsed twice in SDW for 10 s. Samples were air dried and placed on PDA supplemented with penicillin (5 ml/l) and streptomycin (12.5 ml/l). Cultures were incubated in the dark at 22°C for 10 d after which re-isolation of the test fungi was attempted.

**Hemlock phenological trials.** The potential impact of the test fungi on eastern hemlock during the growing season was assessed using 1.5-yr old seedlings (Intervale Conservation Nursery, Burlington, VT) and 4.5-yr old seedlings (Western Maine Nurseries, Fryeberg, ME). Seedlings were transplanted into Metro-Mix 360 in 11.5-, and 23-cm diam pots, for the 1.5 and 4.5-yr old seedlings, respectively, and grown outside prior to treatment.

To assess changes in potential infectivity of the fungi over the growing season, a new group of 40 seedlings (4 plants per treatment and seedling age) were treated each month from June to September for the 1.5-yr old seedlings and May to September for the 4.5-yr old seedlings. Fungal treatments were applied as described above. After treatment, seedlings were incubated in a dew chamber for 24 h at 22 ± 0.5°C and then held in a greenhouse with ambient light and temperature ranging from 9.8 to 24°C depending on the month. Temperature was monitored using HOBO Data Loggers (Onset Computer
Corporation, Bourne, MA). Following treatment, seedlings were inspected monthly for symptoms or signs of disease. A single bioassay was performed during the 2006 plant growing season.

Koch’s postulates were performed 1 mo after treatment as described above, sampling eight needles from a randomly selected twig per seedling. This was repeated at monthly intervals throughout the test period.

Statistical analyses. For the horticultural plant assays, the susceptibility to fungal infection for all test plants was rated according to the probability of re-isolating all fungal strains following treatment. The strawberry plants were used as a reference for infection because this plant is highly susceptible to infection by *C. acutatum* (Horowitz et al. 2004). The frequency of recovering a given fungal strain was also determined for all plant types. The phytopathogenic ERL1380 were selected at random as a reference *Colletotrichum* sp. isolate to which other test isolates were compared.

All data from the bioassays were analyzed using a logistic regression analysis for binary variables. Plant species tested in the bioassays were treated as covariates. Adjusted (log10) odds ratios (with 95% confidence intervals) were calculated using SAS (SAS Institute 1990) and plotted using GraphPad software (Motulsky 1999). A Wald Chi-square test was used to determine significant differences between variables (SAS Institute 1990). Treatments for which the *Fiorinia* strain was not re-isolated were excluded from the odds ratio analysis as zeros in the denominator would result in an undefined number.
**Understory plant screening and molecular identification**

Samples of several common plant species were taken in 10 sites where the *F. externa* epizootic occurred to determine the presence of *C. acutatum* growing in or infecting plants. A total of 97 plants representing 50 species growing in different strata in the hemlock forest were sampled, including low-growing shrubs, vines and trees (Table 4.2). Both live-and dead plant material from the litter were sampled. A 15-20 cm branch or stem sample was taken from each live plant. For the leafy plants, two to three leaves were selected at random, from which four pieces (2 cm$^2$) were excised and placed in individual Petri dishes. For the hemlock needles, eight individual needles (from the litter or live trees) were sampled and placed in separate dishes. All samples were held in an incubator at (22 ± 0.5°C) prior to processing. Samples were surface sterilized as described above and placed on PDA supplemented with penicillin (5 ml/l) and streptomycin (12.5 ml/l), held in the dark at (22 ± 0.5°C) for 7 d and then inspected for the presence of the *Fiorinia* strain. This strain was distinguished from other fungi based on the following characteristics: mycelium and growth medium were pinkish or intensely red, conidia were the typical shape and size for that strain, and the fungus grew faster than other fungi.

To confirm that the *C. acutatum* from the plant material was the entomopathogenic *Fiorinia* strain, a sub-sample of 20 strains recovered from 19 different plant species were identified by molecular methods. DNA was extracted from 1-wk old cultures using the Power Soil$^{\text{TM}}$ DNA kit (Mo Bio Laboratories, Inc., Carlsbad, CA) following the manufacturers’ directions with the following exceptions: 1) Samples were
shaken for 5 min at 5.5 m/s to facilitate breakage of the cell walls using a FastPrep™ FP120 machine (Thermo Savant, Holbrook, NY); 2) DNA was eluted using 100 µl of diluted elution buffer (1:15) (Qiagen, Valencia, CA), and concentrated to 20 µl with a speed vacuum (Eppendorf Centrifuge 5415C, Vaudaux, Schönenbuch, Switzerland). The High Mobility Box (HMG) of the mating-type gene (MAT 1-2) was amplified using primers HMGacuF and HMGacuR (Du et al. 2005).

Polymerase Chain Reaction (PCR) was conducted using Ready-To-Go PCR beads (Amersham Biosciences Inc., Piscataway, NJ) and the following protocol: initial denaturation at 95 °C for 2 min followed by 30 cycles of 95 °C for 30 s (denaturation), 55 °C for 30 s (annealing) and 72° C for 1 min (elongation). PCR products were purified using the QIAquick® PCR purification kit (Qiagen, Valencia, CA) or Princeton Separations Centri Spin™ columns (Princeton, Adelphia, NJ). DNA was stored at 4°C. PCR products were sequenced using a BigDye v3 terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and with the following protocol: 95 °C (initial denaturation) for 3 min followed by 30 cycles of 95 °C for 10 s (denaturation), 50 °C for 5 s (annealing), and 60 °C for 2 min (elongation). PCR fragments were sequenced with a 3130xl Genetic Analyzer (Applied Biosystem, Foster City, CA). Chromatograms were edited and contiguous sequences were generated using Sequencher™ (Gene Codes Corp., Ann Arbor, MI). Sequences generated from this study were compared with MAT 1-2 gene sequences obtained previously for the Fiorinia strain and available at GenBank®. Primers were removed from sequences. Sequences obtained in this study were also deposited in GenBank® (Table 4.4).
Results

Plant bioassays

For all treatments tested, there were no signs of necrosis on plant tissue of pepper, tomato, beans, barley or hemlock. However, endophytic growth of some of the test fungi was detected as indicated by re-isolation of the *Fiorinia* strain from asymptomatic plants after surface sterilization of leaf samples (Figure 4.1A, Table 4.2). Strawberry leaves displayed distinct local necrotrophic symptoms and endophytic growth. Though this plant species appeared to be susceptible to the *Colletotrichum* sp. treatments, the necrotic spots (approx. 1 cm diam) that formed did not progress over the entire leaf nor compromise the viability of the plants 2 mo post treatment (Figure 4.1B).

**Figure 4.1.** (A) Endophytic growth of *Fiorinia* strain EHS<sub>48</sub> in a bean stem 24 h after treatment, *in vitro*, showing germinated germ tubes (a) and appressoria (b); (B) Strawberry with necrotic lesions, 2 mo after spraying with the *Fiorinia* strain.

The percentage of fungal re-isolations per plant species was not significantly different between test years, allowing data across years to be combined (Wald Chi-square = 0.49, *P* = 0.48). Differences in the percentage of plants from which *Colletotrichum* sp.
was re-isolated were significant among the test fungi (Wald Chi-square = 14.72, \( P = 0.03 \)), and a statistically significant 2-way interaction between the test fungi and plant species was detected (Wald Chi-square = 71.02, \( P < 0.0001 \)). Isolate EHS\(_{132}\) (\textit{Lecanicilium lecanii}) and SDW were excluded from the odds ratio analysis because \textit{Colletotrichum} sp. was not re-isolated from these treatments. Approximately 38% of the plants treated with the \textit{Fiorinia} strains (average of \textit{Fiorinia} strains reisolation) became infected based on the re-isolation of \textit{Colletotrichum} sp. (Table 4.2). This was comparable to the phytopathogenic \textit{C. acutatum} from blueberry (31.3%), but significantly different from the \textit{C. acutatum} from tomato, and the entomopathogenic \textit{Orthezia} strain (\( P < 0.05 \)).

\textbf{Table 4.2.} Percentage of fungal re-isolations obtained from the tested plants (N = 8 plants/isolate and plant species). No fungal re-isolations were obtained from control plants treated with SDW and Silwet.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Barley</th>
<th>Beans</th>
<th>Hemlock</th>
<th>Pepper</th>
<th>Strawberry</th>
<th>Tomato</th>
<th>Overall means</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERL(_{1379})</td>
<td>12.5</td>
<td>37.5</td>
<td>12.5</td>
<td>50.0</td>
<td>75.0</td>
<td>0.0</td>
<td>31.3</td>
</tr>
<tr>
<td>ERL(_{1380})</td>
<td>0.0</td>
<td>75.0</td>
<td>0.0</td>
<td>0.0</td>
<td>75.0</td>
<td>0.0</td>
<td>8.3</td>
</tr>
<tr>
<td>ARSEF(_{4360})</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>50.0</td>
<td>0.0</td>
<td>0.0</td>
<td>8.3</td>
</tr>
<tr>
<td>EHS(_{132})</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>EHS(_{41})</td>
<td>25.0</td>
<td>50.0</td>
<td>12.5</td>
<td>37.5</td>
<td>62.5</td>
<td>0.0</td>
<td>31.3</td>
</tr>
<tr>
<td>EHS(_{48})</td>
<td>12.5</td>
<td>50.0</td>
<td>0.0</td>
<td>50.0</td>
<td>75.0</td>
<td>12.5</td>
<td>33.3</td>
</tr>
<tr>
<td>EHS(_{51})</td>
<td>25.0</td>
<td>87.5</td>
<td>0.0</td>
<td>50.0</td>
<td>50.0</td>
<td>25.0</td>
<td>39.6</td>
</tr>
<tr>
<td>EHS(_{58})</td>
<td>25.0</td>
<td>75.0</td>
<td>0.0</td>
<td>37.5</td>
<td>87.5</td>
<td>37.5</td>
<td>43.8</td>
</tr>
<tr>
<td>EHS(_{61})</td>
<td>50.0</td>
<td>75.0</td>
<td>0.0</td>
<td>50.0</td>
<td>75.0</td>
<td>12.5</td>
<td>43.8</td>
</tr>
<tr>
<td>SDW</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Overall means</td>
<td>16.7</td>
<td>50.0</td>
<td>2.8</td>
<td>36.1</td>
<td>55.6</td>
<td>9.7</td>
<td></td>
</tr>
</tbody>
</table>
The mean odds ratio comparing the number of fungal re-isolations from treated plants with the reference, ERL_{1380}, for six of the test fungi were >1, indicating that they were more likely to be re-isolated from the test plants than the reference (Figure 4.2). The odds ratio for the *Orthezia* strain was <1, suggesting that it was less likely to be re-isolated than the other fungi. Because of the variability in the results, differences between this isolate and four of the others were not significant. The odd ratios demonstrated that two of the *Fiorinia* strains were significantly different from the others isolated, indicating that these two were more infective than the reference isolate.

**Figure 4.2.** Adjusted odds ratios (95% confidence interval) of endophytic re-isolations obtained with each fungal inoculation treatment in the plant bioassays. The phytopathogenic *Colletotrichum acutatum* isolate (ERL_{1380}) from a tomato fruit was used as the reference isolate. An odds ratio of 1 indicated no difference between the probability of re-isolating the test fungus and reference strain; ≥1 indicated a greater probability of re-isolating the test strain than the reference strain; ≤1 indicated a greater probability of re-isolating the reference strain than the test strain. Confidence intervals that overlap the reference line (1) are not significantly different ($P = 0.05$).
The mean odds ratios for test plants were all <1, indicating that it was less likely to recover *Colletotrichum* sp. from these plants than the reference species (strawberry) (Figure 4.3). Based on the percentage of plants from which *Colletotrichum* sp. was re-isolated, indicating endophytic fungal growth, beans and strawberries were the most susceptible, while hemlock and tomato were the least (Table 4.2).

![Figure 4.3](image_url)

**Figure 4.3.** Adjusted odds ratios (95% confidence interval) of endophytic re-isolations for each plant tested in the plant bioassays. Strawberry was used as the reference plant. An odds ratio of 1 indicated no difference between the probability of re-isolating the fungus from the test plant species and reference plant; ≥1 indicated a greater probability of re-isolating the fungus from the test plant species than the reference species; ≤1 indicated a greater probability of re-isolating the fungus from the reference plant species than the test plants. Confidence intervals that overlap the reference line (1) are not significantly different (*P* = 0.05).

**Hemlock phenological trials**

A significant statistical effect across months (Wald Chi-square = 12.73, *P* = 0.01) and age of plants (Chi-square = 11.53, *P* < 0.001) for the response of hemlock seedlings
to fungi over the growing season was found. The highest percentage of re-isolations occurred in July among the 1.5 year old seedlings, with between 25-100% recoveries of *Colletotrichum* sp. No fungal re-isolations were recovered from 1.5 and 4.5 yr old hemlock seedlings treated in June, nor in the 4.5 yr old treated in the month of July. Isolate EHS$_{132}$ was never re-isolated from hemlock seedlings and excluded from the analysis (Table 4.3). Based on the odds ratio analysis (Figure 4.4), differences in the rate of fungal recovery among the test isolates were not significant (Wald Chi-square = 2.92, $P = 0.089$).

**Table 4.3.** Percentage of eastern hemlock seedlings from which *Colletotrichum* sp. was re-isolated after treatment (N = 4 seedlings/age and month)

<table>
<thead>
<tr>
<th>Test month/temperature range (°C)</th>
<th>May$^{1,2}$</th>
<th>July (18.3-22.4)</th>
<th>August (14.1-21.2)</th>
<th>September (9.8-18.9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>ER$_L_{1379}$</td>
<td>0</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>ER$_L_{1380}$</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>ARSEF$_{4360}$</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>EHS$_{41}$</td>
<td>0</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>EHS$_{48}$</td>
<td>25</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>EHS$_{51}$</td>
<td>0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>EHS$_{58}$</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>EHS$_{61}$</td>
<td>25</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>SDW</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1 The 1.5-yr old hemlock seedlings were not available for testing in May.

2 With exception of the month of May, temperatures were monitored with HOBO Data Loggers (Onset Computer Corporation, Bourne, MA).
Statistical differences were detected in the susceptibility of the different age of hemlock seedlings tested (Wald Chi-square=11.53, \( P<0.001 \)). The 1.5-yr old hemlock seedlings were more susceptible to infection than the 4.5-yr old seedlings in the months of July and August. In addition, the timing of application influenced infection, with significantly greater likelihood of infection occurring when plants were sprayed in July than the other months tested. No symptoms of infection or negative impact on seedling growth were observed among the test seedlings.

![Figure 4.4](image)

**Figure 4.4.** Adjusted odds ratios (95% confidence interval) of endophytic re-isolations obtained with fungal treatments in the phenological bioassays. The phytopathogenic *Colletotrichum acutatum* isolate (ERL1380) from tomato was the reference isolate. An odds ratio of 1 indicated no difference between the probability of re-isolating the reference isolate and the test fungi; \( \geq 1 \) indicated a greater probability of re-isolating the test strains than the reference isolate; \( \leq 1 \) indicated a greater probability of re-isolating the reference isolate than the test strains. Confidence intervals that overlap the reference line (1) are not significantly different (\( P = 0.05 \)).
Understory plant screening and molecular identification

Of the 97 plants sampled within areas of the epizootic, 37 plants comprising 28 species in 18 families (52% of sampled species), were found to be positive for the presence of *Colletotrichum* sp. (Table 4.4). Twenty of these isolates from four locations where the scale epizootic occurs were molecularly identified as the *Fiorinia* strain (Table 4.4) for the High Mobility Box at the MAT1-2 gene.

Table 4.4. Plant material sampled in epizootic areas and screened for *C. acutatum* (a sample of live leaves from one plant per species was taken unless otherwise indicated)

<table>
<thead>
<tr>
<th>Sample Site</th>
<th>Plant species (family) sampled</th>
<th>C. acutatum recovered</th>
<th>Molecular ID #</th>
<th>GenBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Acer pensylvanicum</em> (Sapindaceae)</td>
<td>Negative</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>A. rubrum</em> (Sapindaceae)</td>
<td>Negative</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td><em>A. pseudoplatanus</em> (Asteraceae) [2 plants]</td>
<td>Negative</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>2, 9, 10</td>
<td><em>A. saccharum</em> (Aceraceae) * [3 trees]</td>
<td>Endophytic</td>
<td>EU043504</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Alliaria petiolata</em> (Brassicaceae) *</td>
<td>Endophytic</td>
<td>EU043511</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>Aralia nudicaulis</em> (Araliaceae)</td>
<td>Endophytic</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>10, 7, 9</td>
<td><em>Aster</em> sp. (Asteraceae)</td>
<td>Endophytic</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td><em>Arisaema triphyllum</em> (Araceae) *</td>
<td>Endophytic</td>
<td>EU043499</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>Barberis thunbergii</em> (Berberidaceae)</td>
<td>Endophytic</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>Betula</em> sp. (Betulaceae)</td>
<td>Negative</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>Catalpa speciosa</em> (Bignoniaceae) *</td>
<td>Endophytic</td>
<td>EU043508</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Carya</em> sp. (Junglandaceae)</td>
<td>Negative</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>Cornus florida</em> (Benthamidida)</td>
<td>Negative</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

(Continue)
<table>
<thead>
<tr>
<th>Sample Site</th>
<th>Plant species (family) sampled</th>
<th>C. acutatum recovered</th>
<th>Molecular ID #</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Cypripedium reginae</em> (Orchidaceae)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>1, 4, 8, 10</td>
<td><em>Dryopteris</em> sp. (Dryopteridaceae)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>Fagus grandiflora</em> (Fagaceae)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>1, 3, 5, 8</td>
<td><em>Fragaria ananassa</em> (Rosaceae)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td><em>Hamamelis</em> sp. (Hamamelidaceae)</td>
<td>Endophytic</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td><em>H. virginiana</em> (Hamamelidaceae) *</td>
<td>Endophytic</td>
<td>EU043501</td>
</tr>
<tr>
<td>1</td>
<td><em>Kalmia latifolia</em> (Ericaceae)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>Liriodendrum tulipifera</em> (Magnoliaceae) * [3 trees]</td>
<td>Endophytic</td>
<td>EU043506</td>
</tr>
<tr>
<td>4, 7, 8</td>
<td><em>L. tulipifera</em> (Magnoliaceae) * [leaves from 5 trees and litter]</td>
<td>Endophytic</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td><em>L. tulipifera</em> (Magnoliaceae) * [from outside epizootic]</td>
<td>Endophytic</td>
<td>EU043516</td>
</tr>
<tr>
<td>1</td>
<td><em>L. tulipifera</em> (Magnoliaceae) * [leaves from 3 trees and litter]</td>
<td>Endophytic</td>
<td>EU043500</td>
</tr>
<tr>
<td>1</td>
<td><em>Maianthemum canadense</em> (Ruscaceae)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td><em>Malus</em> sp. * (Rosaceae) [fruit and leaves]</td>
<td>Signs*</td>
<td>EU043517</td>
</tr>
<tr>
<td>2</td>
<td><em>M. pumila</em> (Rosaceae) *</td>
<td>Endophytic</td>
<td>EU043509</td>
</tr>
<tr>
<td>4</td>
<td><em>Magnolia</em> sp. (Magnoliaceae) *</td>
<td>Endophytic</td>
<td>EU043515</td>
</tr>
<tr>
<td>2</td>
<td><em>Myrica rubra</em> (Myricaceae)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>Onoclea sensibilis</em> (Onicleaceae)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td><em>Pachysandra terminalis</em> (Buxaceae)</td>
<td>Endophytic</td>
<td>-</td>
</tr>
<tr>
<td>2, 10</td>
<td><em>Parthenocissus quinquefolia</em> (Vitaceae) *</td>
<td>Endophytic</td>
<td>EU043505</td>
</tr>
<tr>
<td>2</td>
<td><em>Polystichum acrostichoides</em> (Dryopteridaceae)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>Prunus cerasifera</em> (Rosaceae)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>Sample Site</td>
<td>Plant species (family) sampled</td>
<td>C. acutatum recovered</td>
<td>Molecular ID #</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------------</td>
<td>-----------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>1</td>
<td><em>P. persica</em> (Rosaceae)</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>P. armenica</em> (Rosaceae)</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>P. avium</em> (Rosaceae) *</td>
<td>Endophytic</td>
<td>EU043513</td>
</tr>
<tr>
<td>1</td>
<td><em>Quercus velutina</em> (Fagaceae)</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>Q. americana</em> (Fagaceae)</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>Q. alba</em> (Fagaceae)</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Q. rubra</em> (Fagaceae)</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Rubus sp.</em> (Rosaceae) *</td>
<td>Endophytic</td>
<td>EU043510</td>
</tr>
<tr>
<td>1</td>
<td><em>R. idaeus</em> (Rosaceae)</td>
<td>Endophytic</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>Rosa sp.</em> (Rosaceae)</td>
<td>Endophytic</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>R. multiflora</em> (Rosaceae) *</td>
<td>Endophytic</td>
<td>EU043507</td>
</tr>
<tr>
<td>1</td>
<td><em>Sassafras albidum</em> (Lauraceae) *</td>
<td>Endophytic</td>
<td>EU043498</td>
</tr>
<tr>
<td>2</td>
<td><em>Sorbus americana</em> (Rosaceae) *</td>
<td>Endophytic</td>
<td>EU043512</td>
</tr>
<tr>
<td>2</td>
<td><em>Symlocarpus foetidus</em> (Araceae)</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>Taraxacum sp.</em> (Asteraceae)</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>Thelypteris noveboracensis</em> (Thelypteridaceae)</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Tilia americana</em> (Tiliaceae) *</td>
<td>Endophytic</td>
<td>EU043503</td>
</tr>
<tr>
<td>1</td>
<td><em>Trientalis borealis</em> (Primulaceae) *</td>
<td>Endophytic</td>
<td>EU043502</td>
</tr>
<tr>
<td>9, 10</td>
<td><em>Tsuga canadensis</em> (Pinaceae) [needles from litter]</td>
<td>Endophytic</td>
<td></td>
</tr>
<tr>
<td>9, 10</td>
<td><em>T. canadensis</em> (Pinaceae) [needles from 4 trees]</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>Tussilago farfara</em> (Asteraceae)</td>
<td>Endophytic</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>T. farfara</em> (Asteraceae)</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>Ulmus sp.</em> (Ulmaceae) [2 trees]</td>
<td>Endophytic</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>Vaccinium sp.</em> (Rosaceae) *</td>
<td>Endophytic</td>
<td>EU043514</td>
</tr>
</tbody>
</table>

(Continue)
Sample Site ◊ Plant species (family) sampled | C. acutatum recovered | Molecular ID # GenBank
---|---|---
10 | Verbascum sp. (Scrophulariaceae) | Endophytic | -
9, 10 | Viburnum acerifolium (Adoxaceae) | Endophytic | -
2, 4, 6, 9, 10 | V. acerifolium (Adoxaceae) | Negative | -

* Fungal identification confirmed genetically (High Mobility Box at the MAT1-2, mating type gene).

◊ Location code (sample date): 1 = Pawling Nature Preserve, Dutchess Co., NY (20 June 2006); 2 = South Salem, Westchester Co., NY (10 Nov. 2005; 5, 16, 20, 27 June 2006); 3 = Lawrence Farm, Orange Co., NY (19 June 2006); 4 = Hodgson Farm, Orange Co., NY (19 June 2006); 5 = Whitestone Lane, Rochester City, Monroe Co., NY (13 July 2006); 6 = Poundridge Nurseries, Westchester Co., NY (10 Nov. 2006); 7 = Mountain Lake Park, Westchester Co., NY (10 Nov. 2005); 8 = West Moreland Sanctuary, Westchester Co., NY (11 Jan. 2006); 9 = Olana Historic Site, Columbia Co., NY (29 Aug. 2006); 10 = Macedonia State Park, Litchfield Co., CT (29 Aug. 2006).

These results demonstrate that the *Fiorinia* strain occurs widely within the epizootic areas, growing endophytically within plants, and remaining viable in dead plant material in the litter.

**Discussion**

The research reported herein assessed the host plant associations of several isolates recovered within areas where a fungal epizootic occurs in *F. externa*. The most commonly retrieved fungus from diseased or mummified insects in all sampled sites was molecularly and morphologically identified as *C. acutatum* f. sp. *fiorinia* (*Fiorinia strain*) (Marcelino et al. submitted). This is only the second reported case of a fungus in the genus *Colletotrichum*, a common phytopathogen, causing an epizootic in an insect
pest population. The first was from Brazil where *C. gloeosporioides* f. sp. *ortheziidae* (*Ortheziidae* strain) is causing epizootics in the scale *O. praelonga*, a major pest of *Citrus* spp. (Cesnik and Ferraz 2000). The *Fiorinia* strain may represent a novel means of sustainable biological control for *F. externa* in eastern hemlock forests, but an understanding of their infectivity to plants must be determined before they can be considered environmentally safe.

Plant bioassays were conducted to test the infection potential of several *Fiorinia* strains, the *Ortheziidae* strain, two *C. acutatum* strains and one *L. lecanii*, a known entomopathogen. Several plant species were selected for testing, based on their known susceptibility to *Colletotrichum* spp. The susceptibility of hemlock seedlings was also tested. With the exception of strawberry, no external signs of fungal infection were observed on the plant species tested. However, evidence of *Colletotrichum* sp. growing endophytically was commonly obtained, though variation among plant species was observed. Whereas *Fiorinia* strains were consistently recovered growing endophytically in barley, beans, pepper, strawberry and tomato, only one strain was recovered from hemlock in the plant trials. In contrast, the *Ortheziidae* strain was recovered only from pepper plants.

Strawberry was found to be the most susceptible to infection by the *Fiorinia* strains, exhibiting both external necrotrophic and endophytic growth. However, the necrotic lesions occurred sporadically among test plants, and no negative impact on plant growth was observed. In general the necrotic leaf spots did not exceed 1 cm diam and no conidial masses were produced on the leaf surface after 2 mo. *Colletotrichum* spp.
commonly cause anthracnose disease in strawberries and are a serious concern for the strawberry industry (Horowitz et al. 2004). Non-pathogenic strains of this genus have been reported in strawberries (Horowitz et al. 2002), and it appears that the *Fiorinia* strains may be in this category.

In the hemlock phenological bioassays, the *Fiorinia* strains were commonly recovered from 1.5 yr old seedlings treated in July. The temperature during this month (diurnal average 22°C) may have been ideal for infection and colonization of these strains. These strains and the phytopathogenic *C. acutatum* strains were recovered intermittently at other times of the growing season, but no pattern of infection was observed. Both *C. acutatum* and *C. gloeosporioides* have been reported to affect 70 d old and 4 mo old seedlings of western hemlock, *Tsuga heterophylla* (Raf.) Sarg. (Lock et al. 1984, Hopkins et al. 1985). However, *C. acutatum* f. sp. *pinea* only caused temporary stunting, and the seedlings recovered within the first year (Canadian Food Inspection Agency 1997).

Over 90 different plants representing 50 species were sampled in and around the epizootic areas and processed for infection by *Colletotrichum* sp. *C. acutatum* was recovered from 38% of the plants (37 specimens) sampled comprising 28 species in 18 families which represented a broad range of species in the region. Twenty of the isolates recovered were molecularly identified as the *Fiorinia* strain. These results demonstrate that the *Fiorinia* strain which has been found to infect *F. externa* occurs widely within the epizootic areas, growing endophytically within live and dead plant matter.
With the exception of strawberry plants, only endophytic growth of the *Fiorinia* strains was detected in the plants, some of which are common hosts for species in the genus *Colletotrichum*. We did not observe sporulation either in the field-collected samples or the plants in the bioassays. It is possible that the *Fiorinia* strain, which possesses the capability to infect and kill *F. externa*, may have additional nutritional requirements for its growth and reproduction other than plant tissue alone.

Recently it has been suggested that what are considered specialized monomorphic groups of *Colletotrichum* may constitute distinct lineages of unknown recent origins that may inhabit niches other than plants (Zhu and Oudemans 2000, Guerber and Correll 2001, Peres et al. 2005, Crouch et al. 2006). Our results, which report a *C. acutatum* infecting *F. externa* and causing an epizootic, support this hypothesis. It appears that the *C. acutatum* f. sp. *fiorinia* can inhabit niches other than plants, in this case an insect species. Before the *Fiorinia* strain can be considered for area-wide use against *F. externa* additional research is needed to more fully evaluate its host range, and the conditions under which it causes infection.

**Acknowledgments**

We thank Tom Doubleday, Cheryl Frank, Teri Hata and Dr. Robert Jones for their help with this project. This work was funded in part through a grant awarded by the Northeastern Area State and Private Forestry, USDA Forest Service (#04-CA-11244225286) and is in partial fulfillment of requirements for the PhD degree of J.M. at the University of Vermont.
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CHAPTER 5

Entomopathogenic Activity of a *Colletotrichum acutatum* Strain Recovered from Elongate Hemlock Scale

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¹ Entomology Research Laboratory, The University of Vermont (UVM), Burlington, VT 05405-0105 USA

Abstract

A fungal epizootic in populations of *Fiorinia externa* infesting hemlock trees in forests of the northeastern US has been recently detected. The current known distribution of the epizootic spans 36 sites in New York, Pennsylvania, New Jersey and Connecticut. *Colletotrichum acutatum* f. sp. *fiorinia* was the most prevalent fungus recovered from infected scales. Bioassays indicated that this *Colletotrichum* sp. is highly pathogenic to *F. externa*. Mortality rates of >90 and >55% were obtained for *F. externa* crawlers and settlers, respectively. Other insect species from different orders were bioassayed and mortality of ≤22% was obtained suggesting this fungus may be fairly host specific. This is the second report of a *Colletotrichum* sp. with pathogenic activity towards a scale insect. Our data suggest that *C. acutatum* f. sp. *fiorinia* from *F. externa* epizootics in the US and the previously reported *C. gloeosporioides* f. sp. *ortheziidae* from *Orthezia praelonga* epizootics in Brazil, may constitute distinct biotypes of *Colletotrichum* that have attained the ability to infect insect hosts in addition to the commonly reported plants.

Keywords: *Fiorinia externa*, *Colletotrichum* sp., epizootic, elongate hemlock scale, *Orthezia praelonga*

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Introduction

The eastern hemlock, *Tsuga canadensis* (L.) Carrière, a common species in forests of the northeastern United States is in decline (Orwig et al. 2002). The invasive elongate hemlock scale, *Fiorinia externa* Ferris (Hemiptera: Diaspididae), has been identified as one of the causal agents of this decline (Lambdin et al. 2005). Attempts to control this pest have not been successful. The unique shield-like cover of the scale provides protection from contact insecticides, natural enemies and adverse climatic conditions. Because of its high reproductive rate, even when high mortality exceeding 90% occurs, populations quickly rebound (Baranyovits 1953, Johnson and Lyon 1988).

In 2002 an epizootic was reported within the population of *F. externa* in the Mianus River Gorge Preserve in Bedford, NY (McClure 2002). Sclerotia were found concealing the bodies of adult mummified scales. The origin of the epizootic was unknown, but similar epizootics have been found among scales in 36 different sites in New York, Pennsylvania, Connecticut and New Jersey. A complex of entomopathogenic, phytopathogenic and saprophytic fungi were morphologically and molecularly identified as being associated with the diseased insects (Marcelino et al., submitted). One species, *Colletotrichum acutatum* f. sp *fiorinia* (Marcelino et al., submitted) [hereafter referred to as the *Fiorinia* strain], was dominant in this complex and was consistently recovered in *F. externa* populations in most of the epizootic sites.

Literature on the phytopathogenic genus *Colletotrichum* (Bailey and Jeger 1992, Prusky et al. 2000) includes a single report of the species *C. gloeosporioides* causing significant epizootics in the scale, *Orthezia praelonga* Douglas 1891 (Hemiptera:
Ortheziidae), a major pest of citrus in Brazil. This fungus, *C. gloeosporioides* f. sp. *ortheziidae* is under commercial development for management of *O. praelonga*. (Cesnik et al. 1996). The epizootic caused by the *Fiorinia* strain is the second report of a member of this genus infecting a scale insect. To better understand the role of this fungus in the *F. externa* epizootic, the virulence of the *Fiorinia* strain to four insect species from three orders (Hemiptera, Lepidoptera and Thysanoptera) was evaluated.

**Materials and Methods**

**Isolates.** The virulence of five *Fiorinia* strains from different areas of the *F. externa* epizootic in the Northeast was tested (Table 5.1). In addition, the following other multiple-spored isolates were also assayed: *C. gloeosporioides* f. sp. *ortheziidae* from the Brazilian epizootic in *O. praelonga*, (ARSEF 4360) (obtained from the Agricultural Research Service Entomopathogenic Fungal Collection, Cornell University, Ithaca, NY), two phytopathogenic *C. acutatum*, one isolated from blueberry (ERL 1379) and one from tomato (ERL 1380) (in permanent storage at the Univ. of VT Entomology Research Laboratory (UVM ERL) Worldwide Collection of Entomopathogenic Fungi, Burlington, VT), *Lecanicillium lecanii* (Zimmerman) Gams & Zare (EHS 132), an entomopathogenic strain isolated from *F. externa* and one *Metarhizium anisopliae* (CA-1), recovered from litter in a California avocado orchard (used only in the *Frankliniella occidentalis* bioassays because of its virulence to this insect). Isolates were grown on potato dextrose agar medium (PDA) (39 g/l) supplemented with penicillin (5 ml/l) and streptomycin (12.5 ml/l). Conidia were harvested 10-12 d after inoculation.
Table 5.1. Fungal isolates tested in the insect bioassays

<table>
<thead>
<tr>
<th>Fungus type</th>
<th>Species</th>
<th>Code</th>
<th>Host</th>
<th>Geographic origin</th>
<th>Year of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entomo-pathogenic</td>
<td><em>Colletotrichum</em> f. sp.</td>
<td>EHS₄₁</td>
<td><em>Fiorinia</em></td>
<td>Mohonk, NY</td>
<td>2005</td>
</tr>
<tr>
<td>fungi</td>
<td><em>Fiorinia</em> (Fiorinia</td>
<td></td>
<td><em>Fiorinia</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>strain)</td>
<td></td>
<td><em>externa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Fiorinia</em> strain</td>
<td>EHS₄₈</td>
<td><em>F. externa</em></td>
<td>Bayberry Lane, NY</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td><em>Fiorinia</em> strain</td>
<td>EHS₅₁</td>
<td><em>F. externa</em></td>
<td>Esopus, NY</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td><em>Fiorinia</em> strain</td>
<td>EHS₅₈</td>
<td><em>F. externa</em></td>
<td>Ward Pound Ridge</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reservation, NY</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Fiorinia</em> strain</td>
<td>EHS₆₁</td>
<td><em>F. externa</em></td>
<td>Ward Pound Ridge</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reservation, NY</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Lecanicilium lecanii</em></td>
<td>EHS₁₃₂</td>
<td><em>F. externa</em></td>
<td>South Salem, NY</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td><em>Metarhizium anisopliae</em></td>
<td>CA-1</td>
<td>Soil from</td>
<td>Temecula, CA</td>
<td>2001</td>
</tr>
<tr>
<td>Phytopathogenic</td>
<td><em>C. gloeosporiodes</em> sp. f. <em>ortheziidae</em></td>
<td>ARSEF₄₆₀</td>
<td><em>Orthezia</em></td>
<td>Jaguariuna, Sao</td>
<td>1994</td>
</tr>
<tr>
<td>fungi</td>
<td><em>C. acutatum</em></td>
<td>ERL₁₃₇₉</td>
<td>Blueberry</td>
<td>New Jersey</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>fruit</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>C. acutatum</em></td>
<td>ERL₁₃₈₀</td>
<td>Tomato fruit</td>
<td>Burlington, VT</td>
<td>2005</td>
</tr>
</tbody>
</table>

₈ Used in the *F. occidentalis* bioassays only.
Insects

The virulence of the above fungal isolates was tested against several insect species representing three orders, Hemiptera, Thysanoptera and Lepidoptera, to better understand their comparative infectiveness.

*Bemisia argentifolii* (Hemiptera: Aleroydidae). Silverleaf whitefly was reared on *Bemisia argentifolii* (Hemiptera: Aleroydidae). Silverleaf whitefly was reared on poinsettia (*Euphorbia pulcherrima*) at the UVM ERL according to the protocol of Negasi et al. (1998). For these bioassays, terminal leaves of 2-3 week old bean plants, *Phaseolus vulgaris* (var. Royal Burgundy) were excised and the petiole placed in an Oasis® rooting cube (Smithers-Oasis USA, Kent, OH) held in place by absorbent cotton wool. Cubes were placed in tap water in 9 mm diam Petri dishes until roots formed (~4 d), then 18 mating pairs of whiteflies were removed from poinsettia, anesthetized for 2 s with carbon dioxide and placed on each bean leaf. Infested leaves were held in vented plastic boxes (8.7 cm wide × 9.5 cm long) at 16:8 LD, 75% RH and 24°C. Adults were removed after 24 h to ensure age homogeneity of the progeny. On each leaf 40-180 1st instars were produced.

*Fiorinia externa* (Hemiptera: Diaspididae). Scales were field-collected from understory eastern hemlock trees at the Mount Tom Forest Preserve, Holyoke, MA, which is located outside the known area of the *F. externa* epizootic. One day prior to the bioassay, 30 branches (50 cm long) with new growth and infested with a healthy population of *F. externa* crawlers (i.e., 1st instar mobile nymphal stage emerged from the 3rd instar adult female exuvia) and settlers (i.e., 2nd instar immobile nymphal stage after inserting stylets in the epidermal cells of hemlock leaves, loosing their legs and
remaining anchored for life) were randomly sampled. Branches were kept cool during transport and held at 4°C prior to treatment. Eighty 10 cm long terminal twigs with new growth were clipped from the branches for the assay. On each infested twig a population of 10-200 settlers and 10-46 crawlers were counted. Freshly pruned branches were gathered for each assay repetition.

_Frankliniella occidentalis_ (Thysanoptera: Thripidae). Western flower thrips were reared at the UVM ERL on bean leaves according to the protocol of Doane et al. (1998). Two day old 2nd instars were used for testing. Each replicate of the assay had 10 thrips/leaf.

_Spodoptera exigua_ (Lepidoptera: Noctuidae). Beet armyworm eggs were purchased from Benzon Research, Inc. (Carlisle, PA). Upon delivery, eggs were allowed to hatch in a glass container (12 cm diam. x 20 cm high) containing cabbage leaves. The containers were wiped with an antistatic tissue before introduction of eggs. The glass containers were held for 3-4 d at 22 ± 0.5°C and 16:8 LD. After hatching 20 1st instars were randomly selected for each replicate.

**Bioassays**

For all bioassays, fungal concentrations of $10^6$ and $10^7$ conidia/ml$^{-1}$ suspended in sterile distilled water (SDW) and 0.02% Silwet were tested. A suspension of SDW and 0.02% Silwet was used for the controls in the _F. externa_ trials. SDW served as the controls for the other test insect species. Each insect bioassay was repeated three times with four replicates for each treatment.
For the whitefly assays, each treatment consisted of four leaves, each with 40-180 2 d old 1st instars. A Potter Precision Laboratory Spray Tower (Burkard Manufacturing Co. Ltd., Rickmansworth, Hertfordshire, UK) operating at 0.84 kg/cm² with a 0.25 mm diam nozzle was used to spray 2.5 ml of the fungal suspension. Mortality was assessed after 30 d. Individual insects were first inspected for morphological changes in the cuticle or body, i.e., changes in color or body turgor. In cases where mortality could not be confirmed visually, a fluid sample from the hemolymph was mounted on a glass slide and the cell structures were observed for evidence of disruption or death, as compared with that from live, healthy individuals.

For the *F. externa* assays, isolates were tested against settlers and crawlers using modified protocols of Rose (1990) and Butt & Goettel (2000). For each treatment, four twigs, each containing ≥10 crawlers or settlers, were held vertically in a metal test tube rack and individually sprayed at a distance of 38 cm with a micro droplet mist of 250 µl of conidial suspension using a hand-held plastic spray bottle. Twigs were allowed to air dry for 2 min, then placed individually in sterile graduated 50 ml conical plastic tubes (Quikrete, Atlanta, GA) containing 16 g of sterilized sand and 7 ml SDW. The sand served to hold the twigs upright and furnished water to prevent desiccation and maintain a humid environment. Each tube was covered loosely with a cap which allowed ventilation. Tubes were placed in plastic bags to prevent desiccation and held at 22°C with 16:8 LD. Mortality of crawlers and settlers was determined 21 d after treatment as described above for the whitefly assay.
For the thrips assays, bean leaf discs (3.3 cm diam) were placed on moist filter paper in 3.5 cm diam Petri dishes, to which 10-2\textsuperscript{nd} instars were added. Each Petri dish was sprayed with 2 ml of the fungal suspension using a Potter Spray Tower, as described previously. After being air dried for 2 min, Petri dishes were covered and sealed with parafilm, and held in the dark at 22 ± 0.5°C. Mortality was assessed 7 d after treatment. Insects that exhibited obvious signs of fungal infection, i.e., displayed an abnormal body color or lacked turgor, and those that did not respond when gently probed with a small insect pin were considered dead.

Beet armyworms were assayed in well plates with 5 x 4 cells (13 mm diam/cell) (Model #BIO-BA-128, C-D International, Pitman, NJ). A 10 mm diam disc of moist filter paper was placed in the bottom of each well, followed by 10 mm diam cabbage leaf disc and one 1\textsuperscript{st} instar beet armyworm. Each 20-cell unit was sprayed with 2 ml of the test suspension with a Potter Spray Tower. Cell units were air dried for 2 min, covered with clear plastic wrap and held in an incubator in the dark at 22 ± 0.5°C. Mortality was assessed after 7 d as described for the thrips assays.

To verify Koch’s postulates (i.e., re-isolation of the test fungi from a diseased host after treatment of a healthy individual), a random subsample of 10 insects from each treatment was taken. Each insect was surface sterilized in 0.01% bleach, rinsed in SDW and placed in a Petri dish on PDA with 5 ml/l penicillin and 12.5 ml/l streptomycin. Petri dishes were held in the dark at 22 ± 0.5°C for 7 d, and then cadavers were examined for the presence of the \textit{Fiorinia} strain or the other test strains.
Statistical analyses

Because protocols differed among the insect species bioassayed, statistical analyses of mortality were made separately for each species. Transformation of the data was not required as they were normally distributed. Variances were not homogeneous (using Levene's test), which required use of a Welch’s one way ANOVA (unpooled variances). An adjusted pairwise comparison between fungal isolates within a test insect species was made using a post-hoc Tukey-Kramer test. The effect of suspension concentration (10\(^6\) or 10\(^7\) conidia/ml\(^{-1}\)) was determined with an adjusted least square means (LS means). \(P < 0.05\) was considered statistically significant. All statistical analyses were performed using SAS® (SAS Institute 1990) and plotted using SPSS® (SPSS Inc. 2005).

Results

Definite symptoms and signs of infection were observed among the insects treated with the *Fiorinia* strains, demonstrating its entomopathogenic capacity (Figure 5.1). Mortality varied depending on isolate, conidial concentration and insect species tested (Table 5.2 and 5.3).

The mean percent mortality among *F. externa* crawlers and settlers treated with the *Fiorinia* strains ranged from 78-90% and was significantly greater than mortality for all of the other test isolates and the blank control (\(P < 0.05\)) (Figure 5.2). In general mortality from the *Fiorinia* strains was about 30% greater among the crawlers than the settlers. For the bioassay with crawlers, the mortality obtained from the two other known entomopathogens, the *Ortheziidae* strain and EHS\(_{132}\), was not significantly different from
one of the plant pathogens, ERL\textsubscript{1379}. Mortality following treatment with the other plant pathogen, ERL\textsubscript{1380}, was significantly greater than the two entomopathogenic strains. These results demonstrate that other \textit{C. acutatum} strains, known to be plant pathogens have the potential to infect insects, but their virulence was not consistent within or between insect species. Differences in mortality between the two conidial concentrations were not significant for any of the isolates tested against \textit{F. externa} for crawlers and settlers.

\textbf{Figure 5.1.} Fungal structures from bioassays of the \textit{Fiorinia} strain and symptomatic and non-symptomatic \textit{F. externa}. A) acervuli arising from head of an infected whitefly; B) Crawler with septicemia from EHS\textsubscript{51}; C) septicemia in \textit{F. externa} adult from EHS\textsubscript{41}; D) generalized septicemia in crawlers from EHS\textsubscript{51} treatment; E) arrested development in diseased \textit{F. externa}; F) normal development of the scale cover in \textit{F. externa} from controls; G) normal development of the scale cover in nature; H) mycelium arising from adult \textit{F. externa}. 
Table 5.2. Results of statistical analysis for bioassays with *Fiorinia externa* immatures

<table>
<thead>
<tr>
<th>Main Effect (Welch’s one way ANOVA)</th>
<th>F. <em>externa</em> settlers (N=12,343)</th>
<th>F. <em>externa</em> crawlers (N=989)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td><strong>Main Effect</strong></td>
<td>33.19</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Test isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHS41</td>
<td>0.75</td>
<td>0.38</td>
</tr>
<tr>
<td>EHS48</td>
<td>0.00</td>
<td>0.97</td>
</tr>
<tr>
<td>EHS51</td>
<td>0.51</td>
<td>0.47</td>
</tr>
<tr>
<td>EHS58</td>
<td>1.12</td>
<td>0.29</td>
</tr>
<tr>
<td>EHS61</td>
<td>0.03</td>
<td>0.86</td>
</tr>
<tr>
<td>ERL1379</td>
<td>1.20</td>
<td>0.27</td>
</tr>
<tr>
<td>ERL1380</td>
<td>0.01</td>
<td>0.92</td>
</tr>
<tr>
<td>EHS132</td>
<td>0.01</td>
<td>0.93</td>
</tr>
<tr>
<td>ARSEF4360</td>
<td>0.07</td>
<td>0.79</td>
</tr>
<tr>
<td>Blank</td>
<td>0.29</td>
<td>0.59</td>
</tr>
</tbody>
</table>

* Significant differences in percent mortality.

* Not tested.
Table 5.3. Statistical analysis for bioassays with *Spodoptera exigua*, *Bemisia argentifolii* and *Frankliniella occidentalis*

<table>
<thead>
<tr>
<th>Test isolates</th>
<th>EHS41</th>
<th>&lt;0.001</th>
<th>1*</th>
<th>3.87</th>
<th>0.05</th>
<th>1</th>
<th>1.44</th>
<th>0.23</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHS48</td>
<td>17.67</td>
<td>&lt;0.001</td>
<td>1*</td>
<td>2.21</td>
<td>&lt;0.13</td>
<td>1</td>
<td>0.00</td>
<td>1.00</td>
<td>1</td>
</tr>
<tr>
<td>EHS51</td>
<td>20.28</td>
<td>&lt;0.001</td>
<td>1*</td>
<td>1.19</td>
<td>&lt;0.27</td>
<td>1</td>
<td>0.36</td>
<td>0.54</td>
<td>1</td>
</tr>
<tr>
<td>EHS58</td>
<td>0.81</td>
<td>0.368</td>
<td>1</td>
<td>6.49</td>
<td>&lt;0.01</td>
<td>1*</td>
<td>3.24</td>
<td>0.07</td>
<td>1</td>
</tr>
<tr>
<td>EHS61</td>
<td>0.36</td>
<td>0.543</td>
<td>1</td>
<td>6.45</td>
<td>&lt;0.01</td>
<td>1*</td>
<td>1.44</td>
<td>0.23</td>
<td>1</td>
</tr>
<tr>
<td>ERL1379</td>
<td>1.44</td>
<td>0.231</td>
<td>1</td>
<td>1.56</td>
<td>&lt;0.21</td>
<td>1</td>
<td>1.44</td>
<td>0.23</td>
<td>1</td>
</tr>
<tr>
<td>ERL1380</td>
<td>3.24</td>
<td>0.073</td>
<td>1</td>
<td>4.32</td>
<td>&lt;0.03</td>
<td>1*</td>
<td>5.76</td>
<td>0.01</td>
<td>1*</td>
</tr>
<tr>
<td>EHS32</td>
<td>70.67</td>
<td>&lt;0.001</td>
<td>1*</td>
<td>7.41</td>
<td>&lt;0.007</td>
<td>1*</td>
<td>0.00</td>
<td>1.00</td>
<td>1</td>
</tr>
<tr>
<td>ARSEF4360</td>
<td>0.00</td>
<td>1.00</td>
<td>1</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
</tr>
<tr>
<td>CA-1</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>81.03</td>
<td>&lt;0.0001</td>
<td>1*</td>
</tr>
<tr>
<td>SDW</td>
<td>3.24</td>
<td>0.073</td>
<td>1</td>
<td>0.01</td>
<td>0.93</td>
<td>1</td>
<td>0.36</td>
<td>0.54</td>
<td>1</td>
</tr>
</tbody>
</table>

* Significant differences in percent mortality.

• Not tested.
Figure 5.2. Percent mortality among *F. externa* settlers and crawlers in fungal bioassays. (± 95% CI of the mean). Bars with the same letters are not significantly different (data for both concentrations combined) \((P < 0.05)\) using the post-hoc multiple comparison Tukey-Kramer test.

Mortality of \(\leq 22\%\) was obtained for all of the other insect species tested \((P < 0.05)\) (Figure 5.3). For some of the test isolates, significantly greater mortality was obtained from the high concentration, though no consistent pattern was observed across insect species or isolates. Of all the insect species tested, whiteflies appeared to be more susceptible to infection at the higher concentration than the other insects. Whiteflies treated with the plant pathogenic strains of *C. acutatum* exhibited mortality rates comparable with those of the *Fiorinia* strains.
**Figure 5.3.** Percent mortality among different test insect species in fungal bioassays. (± 95% CI of the mean). Bars with the same letters are not significantly different (data for both concentrations combined) \( P < 0.05 \) using the post-hoc multiple comparison Tukey-Kramer test. 
- Statistical significance not estimated for this isolate because data for only one concentration was collected.
Koch’s postulates were successfully achieved for the *Fiorinia* strains in silverleaf whitefly, *F. externa* and western flower thrips. In addition, whereas *F. externa* in the control treatments and those sprayed with *C. gloeosporioides* f. sp. *ortheziidae* underwent normal development and reached maturity (Figure 5.1F-G), normal development was halted among *F. externa* treated with the *Fiorinia* strains and phytopathogenic *C. acutatum*. Infected *F. externa* settlers did not attain maturity (Figure 5.1E) and settlers and crawlers showed symptoms of mycosis (Figure 5.1D-E).

**Discussion**

This research clearly demonstrates that the *Fiorinia* strains, isolated originally from *F. externa* from several sites where a fungal epizootic has been observed, is highly pathogenic to that host, particularly in the crawler stage. In addition Koch’s postulates were satisfied as these test isolates were recovered from cadavers proving that they were killed by infection from these fungi. Infection was also obtained when *F. externa* crawlers and settlers were treated with other *C. acutatum* isolates known to be phytopathogenic, though mortality rates were significantly lower than those obtained from the epizootic. In contrast, other insects from the same and different orders displayed comparably lower susceptibility to both the entomopathogenic and phytopathogenic *Colletotrichum* isolates.

The present report of the *Fiorinia* strain and its virulence to *F. externa* along with the research demonstrating the infectivity of *C. gloeosporioides* f. sp. *ortheziidae* to scales of citrus supports the hypothesis that members of the genus *Colletotrichum* have a broader host range and niche than what has currently been reported (Guerber and Correll
Considering the common occurrence of epizootics linked with the *Fiorinia* strain in populations of *F. externa* within hemlock forests in the Northeast, this can not be considered an unusual occurrence that takes place under very specific conditions. Based on the bioassays representing only a few insect species, it appears that this host range extension by the *Fiorinia* strain is limited primarily to scale insects, or perhaps to Hemiptera in general. Samples of other insects occurring within the epizootic have been sampled, representing a wide variety of species and orders, yet no evidence of infection by the *Fiorinia* strain in these specimens were obtained (J.M., unpublished data).

Many questions regarding this unique phenomenon remain that must be answered before exploitation of this potential biological control agent can be considered. Extensive sampling within *F. externa* populations in and outside the epizootic area is needed to determine the geographical range of the *Fiorinia* strain. This would provide essential information on its current distribution, its rate of expansion and dispersal, and the site of origin. Currently it is unknown if this entomopathogenic strain occurred here before the accidental introduction of *F. externa* or arrived in association with the scale. It is possible that in its native habitat, this fungus maintains it naturally at low levels. The process by which infection of this fungus takes place also requires further study. The *Fiorinia* strain has been found growing endophytically in over 28 different species of plants within the epizootic areas, though external evidence of infection (signs and symptoms) is lacking (J.M., unpublished data). It is unknown if *F. externa* becomes infected through contact with these plants or some other means. This research shows many aspects of fungal
ecology are only now being discovered, even for organisms such as *Colletotrichum* which has been studied extensively for over a century.

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CHAPTER 6

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The Hyphomycete genus *Aschersonia*, named by Montagne (1848) is the conidial stage of the teleomorph *Hypocrella* (Clavicipitaceae). Most of known species occur in the *Aschersonia* form. Perithecia are rarely collected (Mains, 1959a). The great majority of species were initially described as having a pantropic distribution (Thaxter, 1914). *Hypocrella* spp. were first reported in Cuba, Puerto Rico, Paraguay and the Philippines in the leaf surface of different plant species in the early 20th Century (Seaver, 1920). Although the genus was described as parasitic in plants, it was later established that it is parasitic on insects in the Hemipteran families Coccidae (soft scales), Diaspididae (armored scales) and Aleyrodidae (whiteflies), infesting the plants where they are found (Petch, 1921). Species of this genus have been used as a biological control for Japanese citrus scale, *Lopholeucaspis japonica* (Cockerell) in the Black Sea coast of Georgia (Tabatadze and Yasnosh, 1997; Yasnosh and Tabatadze, 1997; Tabatadze and Yasnosh, 1999).

Until 1919, 60 species had been described (Petch, 1921). However, only 54 scientific names covering 42 species were valid. In the species group parasitic to scale insects, Petch (1921) stated there were 22 species of *Hypocrella*, with the corresponding *Aschersonia* anamorph being known in 12 cases. Three unattached species of *Aschersonia* (teleomorph not known) where also listed. For whiteflies, there were 13 species of *Hypocrella* of which anamorph *Aschersonia* stages were known, and 14 unattached species of *Aschersonia* (Steinhaus, 1949). Petch (1925) was able to examine a significant portion of all herbarium material in existence around the world and estimated
that there were 25 *Aschersonia* spp. and 29 *Hypocrella* spp. Apart from the work developed by Petch, few taxonomical studies have been conducted, with the exception of Mains (1959b) who reviewed the number of *Aschersonia* species, reporting the existence of seven *Aschersonia* spp. occurring in North America. Currently, this genus is lacking an extensive taxonomical revision and Evans and Hywel-Jones (1997) noted that many species from tropical forest ecosystems remain to be described.

Members of *Aschersonia* have a superficial stroma (when present, on the lower surface of the leaves), no hyphae penetrating the leaf, generally white or brightly colored in various shades of yellow, red or brown turning black with age in most species, and a subglobose, hemispherical or pulvinate shape (Petch, 1914, 1921). The conidia spores are usually ellipsoid, granular and not septated, although variations in the shape have been reported. Spores are born singly on short filiform hyphae (Samson and McCoy, 1983). In early stages of infection, the fungus develops internally within the host and thus not visible externally. Soon, a dense marginal fringe is formed by the hyphae bursting out from the interior around the edge of the host (Fawcett, 1948). In its mature stage the fungus completely covers the insect which in some cases makes it difficult to identify the parasitized hosts. Cross sections of the stroma may not show recognizable remnants of the host (Samson and McCoy, 1983), hence the past assumption that the fungus was parasitic on leaf tissue.

Webber (1894, 1897) first reported the entomogenous condition of the anamorph *Aschersonia* while conducting investigations in entomopathogenic fungi on citrus trees. Gossard (1903) and Fawcett (1948) continued Webber’s work. Since 1904, species of *Aschersonia* have been described as occurring on scale insects (Petch, 1921), especially
in the tropics, but also present in temperate regions (Australia, Africa, India, Java, New Zealand and North America) attacking the insects in epizootic patterns (Parkin, 1906).

The successful use and establishment of several *Aschersonia* spp. (mainly *Aschersonia aleyrodis* Webb.; *Aschersonia goldiana* Sacc. and Ellis and *Aschersonia placenta* Berk. et Br.) for control of greenhouse whiteflies (Hemiptera: Aleyrodidae) has been reported in North America (Fawcett, 1908a,b, 1948; Berger, 1919, 1921, 1944; McCoy, 1985), Germany (Hirte et al., 1989), The Netherlands (Fransen 1987, 1995, Fransen and Van Lenteren, 1993, 1994, Meekes et al. 2000, 2002), eastern Europe (Ponomarenko et al., 1975; Solovei, 1976; Spassova, 1980; Shutova et al., 1985), Colombia (Sarmiento et al., 1995) and Asia (Zhu-An and Qing-Tao, 1986). As a result of the successful biocontrol achieved with *Aschersonia* spp. in citrus pests, its use has been extended to cucumber and tomato whitefly pests (Spassova et al., 1980; Ramakers and Samson, 1984). The toxigenic properties of metabolites from *Aschersonia* spp. have also been studied (Krasnoff and Gibson, 1996; Boonphong et al., 2001, Watts et al., 2003).

*Aschersonia* spp. have been extensively studied for the biological control of whiteflies. The potential use of *Aschersonia* for management of scale insects has been neglected considering the relevant importance of some species of Diaspidids. Research within this area urges since to accomplish effectiveness, several factors must be equated, including assessing potential, efficacy, dissemination, persistence, compatibility with other regulatory agents, and establishment in the host population (see Fuxa, 1987; Jaques, 1983).