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Survival and dissemination of conidia of Discula destructiva Redlin through the alimentary canal of an insect

Bryan E. Hed

To the Graduate Council:

I am submitting herewith a thesis written by Bryan E. Hed entitled "Survival and dissemination of conidia of Discula destructiva Redlin through the alimentary canal of an insect." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Mark T. Windham, Major Professor

We have read this thesis and recommend its acceptance:

Jerome Grant, Alan Windham

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate
School

SURVIVAL AND DISSEMINATION OF CONIDIA OF *Discula destructiva* Redlin THROUGH THE ALIMENTARY CANAL OF AN INSECT

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Bryan E. Hed

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ABSTRACT

Discula destructiva Redlin, the cause of dogwood anthracnose on *Cornus florida* L., is known to be spread by wind and rain. The rapid spread of this pathogen suggests that insects also may be important in the dissemination of *D. destructiva*. Viable conidia of *D. destructiva* can be carried externally and internally by convergent lady beetles (CLBs), *Hippodamia convergens* Guerin-Meneville. It is not known if conidia carried internally are discharged in viable condition, and if so, are capable of infecting susceptible hosts. Using CLBs as a model insect, the objectives of this research were to: 1) develop a nonlethal method of surface-disinfesting adult CLBs, 2) determine if the beetles can discharge viable conidia of *D. destructiva* in frass, and if so, for how long, 3) quantify the amount of conidia in frass, and 4) determine if the beetles, exposed to conidia of *D. destructiva* and then surface-disinfested, can initiate infection of healthy dogwood leaves in the greenhouse, by discharging viable conidia of *D. destructiva* in frass.

Beetles were surface-disinfested after exposure to *D. destructiva* to ensure that their frass would not be contaminated by conidia carried externally. Seven different durations of disinfestation were tested (0 s, 15 s, 30 s, 45 s, 60 s, 2 min, and 5 min), using a solution of 0.525 % sodium hypochlorite and 5 % ethanol. Disinfestation of the beetles was always followed by a 15 s rinse in sterile water. Ten percent of the water used to rinse the beetles was tested for viable conidia. The 5 min disinfestation was used as the standard disinfestation period for all subsequent experiments because it was the longest disinfestation period that resulted in ≤ 50% beetle mortality and the absence of viable

propagules of *D. destructiva* in the beetle rinse water. Mortality caused by the 5 min disinfestation averaged 24% for all subsequent experiments.

To determine if conidia of *D. destructiva* were discharged in frass in viable condition, beetles were exposed to sporulating cultures of *D. destructiva* for 1 h and then surface-disinfested. Frass was collected after disinfestation and tested for the presence of viable conidia. Seventy-six percent of the beetles tested discharged viable conidia of *D. destructiva* in their frass. More than 80%, 32%, 19%, 13%, and 12% of the beetles from which *D. destructiva* was isolated retained viable conidia of *D. destructiva* internally for at least 12, 24, 48, 72, and 96 h, respectively. A hemacytometer slide was used to quantify conidia of *D. destructiva* in frass. The numbers of conidia varied greatly, ranging from 0 to 3.2×10^6 conidia/frass pellet.

In a greenhouse inoculation chamber, conidia of *D. destructiva* discharged in frass of CLBs were capable of infecting susceptible dogwood leaf surfaces. Sixty-eight percent of branches treated with beetles exposed to *D. destructiva* and 32% of branches treated with beetles exposed to *D. destructiva* and then surface-disinfested became infected with *D. destructiva*. No infection by *D. destructiva* occurred on branches with non-infested beetles and no beetles (controls).

Insects may contribute to the spread of dogwood anthracnose. A variety of insects can acquire and transport viable conidia of *D. destructiva* externally in the field. These results suggest that insects also may pass viable conidia of *D. destructiva* in their frass and thereby inoculate leaves of flowering dogwood resulting in symptoms of dogwood anthracnose.

TABLE OF CONTENTS

LIST OF TABLES

TABLE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

CHAPTER I

LITERATURE REVIEW

Flowering dogwood, *Cornus florida* L., is an important native understory tree found extensively throughout forests of the eastern United States. Its fruit, foliage, and twigs are a rich source of nutrition, especially calcium, for many species of birds and wildlife (Mitchell et. al. 1988). The high calcium concentration and rapid decomposition of its leaves act to build soil fertility in the forest (Thomas 1969).

Aside from its ecological significance, flowering dogwood also is an important component of urban landscapes as its showy bracts in spring are of exceptional ornamental quality. More than 100 commercial cultivars have been developed (Santamour and McArdle 1985) due to its popularity, and it has become one of the most important crops of the nursery industry in Tennessee. Sales of flowering dogwood have generated 30-40 million dollars annually for Tennessee nurseries, and Tennessee produces approximately 75% of all dogwoods sold in the United States. The dogwood's ornamental quality is important to tourism and has made it a favorite element in festivals throughout the south. A Dogwood Arts Festival is held every April in Knoxville, Tennessee, generating millions of dollars in revenue each year (Southards 1995).

In the late 1970s, a lethal anthracnose-like disease was reported on flowering dogwood, affecting thousands of trees in southeastern New York and southwestern Connecticut. The 'mysterious disease' was initially attributed to *Colletotrichum*

gloeosporioides (Penz) Penz. and Sacc., and the epidemic was attributed to environmental conditions highly conducive to fungal infection and stress in the trees (Pirone 1980).

By 1983, experts recognized that this new disease on flowering dogwood was caused by a fungus of the genus *Discula* (Daughtrey and Hibben 1983). A similar disease also associated with species of *Discula* (Salogga and Ammirati 1983), had been described on western flowering dogwood, *Cornus nuttallii* Aud., in the Pacific Northwest and had been given the name dogwood anthracnose (Byther and Davidson 1979). Comparisons of symptoms, signs and isolates of the causal fungus confirmed that the same disease occurred on both coasts (Hibben and Daughtrey 1988).

This new species of *Discula* was described in 1991 and is now known as *Discula destructiva* Redlin. *Discula destructiva* can be distinguished from other *Discula* species by the morphology of the conidiogenous cells which have narrow accuminate apices (Redlin 1991). The subcuticular acervuli are produced in abundance and are most often directly beneath leaf trichomes, a feature characteristic of infection by this fungus (Redlin 1992). Under conditions of high humidity, conidia ooze from acervuli in a mucilaginous matrix. No sexual stage of this fungus has been found (Daughtrey et al. 1996).

Another undescribed species of *Discula* also has been associated with symptoms of dogwood anthracnose and was isolated in 7 to 8% of the cases of anthracnose in a study from native dogwoods in Pennsylvania and Maryland (Trigiano et al. 1995a, 1995b). Although causing identical symptoms on flowering dogwood, *D. destructiva* can be distinguished from the undescribed *Discula* spp. by its ability to oxidize gallic acid, a polyphenol, to quinones (Trigiano et al. 1995a, 1995b).

Genetic analysis has provided information suggesting that *D. destructiva* is not native to North America. DNA amplification fingerprinting of 28 isolates of *D. destructiva* from eastern and western United States and western Canada revealed a highly conserved genome throughout the range of the disease. This lack of genetic diversity and the rapid spread and devastation caused by this pathogen suggest that *D. destructiva* has been recently introduced into North America (Trigiano et al. 1995a, 1995b). Fingerprint analysis of three isolates of the undescribed *Discula* spp. on dogwood, revealed a greater range of genetic diversity more characteristic of an indigenous species (Trigiano et al. 1995a, 1995b). Evidence from phylogenetic analysis of *D. destructiva* and other *Discula* species (*D. umbrinella, D. campestris, D. fraxinea, D. platani, D. quercina*, and the undescribed *Discula* spp. on dogwood) revealed that *D. destructiva* isolates were more closely related to *D. umbrinella, D. fraxinea,* and *D. campestris* (European tree pathogens) than *D. quercina* and the undescribed *Discula* spp., considered native to North America (Caetano-Anolles et al. 1997a, 1997b). Studies involving the genetic relatedness of double-stranded RNAs using a dot blot hybridization procedure and arbitrary signatures from amplification profiles have suggested that isolates of *D. destructiva* collected from the Pacific North West and western Canada have a different origin from isolates collected from the eastern United States, and that these represent separate introductions (Yao et al. 1997, Caetano-Anolles et al. 1997b).

Dogwood anthracnose spread rapidly throughout the Appalachian mountain region. In 1983, the disease was reported in New York, Connecticut, Pennsylvania and New Jersey (Daughtrey and Hibben 1983) and in Catoctin Mountain Park in Maryland (Mielke and

Langdon 1986). By 1987, it had been reported in four additional eastern states (Delaware, Massachusetts, Virginia, and West Virginia) and as far south as northern Georgia. The following year it was found in North Carolina, South Carolina, and Tennessee, and in 1989, Kentucky and Alabama (Anderson et al.1991). At present the disease also can be found scattered throughout several midwestern states, often from the movement of infected plant material (Daughtrey et al. 1996, Hartman 1997, Ruhl 1997, Schwegman 1996, Taylor 1997).

The spread of dogwood anthracnose through the southern Appalachians has slowed and is not expected to spread farther south than its present distribution (Britton 1997). However, the epidemic has taken its toll. In North Carolina alone, 217 million dogwoods are either infected by or have died from dogwood anthracnose (Rogers 1997). In Catoctin Mountain Park, Maryland, flowering dogwoods were reduced 77% throughout the park from 1976 to 1992, and 94% in dogwood anthracnose impact plots (where dogwoods were prevalent) from 1984 to 1994 (Sherald et al. 1996). An impact assessment study composed of 210 plots in seven southeastern states revealed that the hectarage with diseased dogwoods had increased from 0.2 million (0.5 million acres) in 1988 to 7 million (17.3 million acres) by 1993 (Knighten and Anderson 1993, Daughtrey et al. 1996). Dogwoods monitored in 38 permanent plots in Great Smoky Mountains National Park showed a 638% increase in the number of plots with severe anthracnose epidemic from 1988 to 1992 (Windham et al. 1993). From observations in the Mohonk Preserve in New Paltz, New York, only 11% of the original population of dogwoods remained in 1991, 10 years after the disease had been introduced (Daughtrey et al. 1996). Dogwood

anthracnose will probably eliminate flowering dogwood from elevations above 1500 m in the Appalachian mountains (R. Anderson, personal communication). *Discula destructiva* also has caused extensive mortality of *C. nuttallii* in the Pacific Northwest and Idaho. However, it has not aroused the level of concern in the west as it has in the east on *C. florida* partly because *C. nuttallii* is not as prized as an ornamental in the west as *C. florida* is in the east (Daughtrey et al. 1996).

Discula destructiva can attack leaves, flowers, twigs, branches (Hibben and Daughtrey 1988), fruit, and seeds (Britton et al. 1993). Symptoms include severe leaf blight, twig and branch death. Trees in shaded locations often die in 1 to 5 years (Hibben and Daughtrey 1988). Dieback often begins with the lower branches, progressing upward, especially in relatively exposed locations. Understory trees with shaded canopies are more uniformly affected (Daughtrey et al. 1996). The leaves develop purple rimmed spots and blotches with tan or brown centers that sometimes fall out producing shot holes. Blotches often begin at the leaf tip and expand in a wedge shaped pattern down and from the midrib or between veins until the entire leaf is dead. The fungus can enter twigs via leaf petioles or direct penetration. Blighted leaves may remain on the tree through winter and are a source of inoculum in spring. Diseased trees often sprout epicormic shoots along the main branches and truck. This succulent growth is rapidly colonized by the fungus and can provide a direct pathway to the trunk, leading to trunk cankers (Hibben and Daughtrey 1988).

Environment plays a major role in the severity of dogwood anthracnose. Trees growing in full sun with adequate moisture are least at risk whereas trees growing in dense

shade and stressed by drought are most at risk (Erbaugh 1992). Lesions on shaded leaves expand more rapidly than lesions on leaves in full sun (Windham et al.1995). Disease incidence and severity are greater in canopies of understory trees where evaporative potential is low (Chellemi and Britton 1992). Rainfall plays an important role in disease development as symptom development begins during cool wet weather in spring, when leaves are first expanding (Hibben and Daughtrey 1988). High precipitation levels are a key element in the development of epidemics as wet conditions are conducive to sporulation of the fungus (Daughtrey et al. 1996). Rainfall pH may affect disease severity. In a study with simulated acid rain applied to leaves before inoculation, disease incidence and severity increased as pH decreased from 5.5 to 2.5 (Anderson et al. 1993). Acid rain causes cracking, dessication and erosion of leaf cuticle surrounding trichomes and may predispose dogwoods to anthracnose (Brown et al. 1994).

Topographic features of dogwood habitat may affect disease incidence and severity as they affect temperature and moisture levels. In Great Smoky Mountains National Park, trees within 50m of streams were more likely to suffer severe anthracnose symptoms than trees located more than 50 m from water. Near water, understories and overstories may tend to be more dense, have higher humidity, and lower light intensity with respect to dogwood canopies. Such conditions are conducive to sporulation of *D. destructiva* (Windham et al. 1991). Dogwoods growing on north facing slopes had more disease than those on south, east, or west facing slopes (Windham et al. 1991), where higher light intensity and higher leaf temperatures (Windham et al. 1995) and greater evaporative demand (Chellemi and Britton 1992) were less conducive to lesion growth and

reproduction by the fungus.

Although the spread of dogwood anthracnose has been well documented, relatively little research has been done on the dissemination of *D. destructiva*. Exudation of the conidia in a sticky matrix suggests conidial dispersal by rain splash, birds and insects (Daughtrey and Hibben 1994). The use of spore traps has shown that conidia of *D. destructiva* are wind-spread during rainy periods (Daughtrey et al. 1988). But wind and rain do not fully account for the rapid spread of *D. destructiva* through the Appalachian mountains (Grant et al. 1995). The fungus has been isolated from dogwood seeds and fruit of infected trees, making migratory birds possible vectors of *D. destructiva* over long distances (Britton et al. 1993).

Insects have been implicated in the spread of numerous destructive fungal pathogens of trees. Amylostereum rot, a white rot of larch, fir and pine is spread by wood wasps of the family Siricidae. Oidia carried by the female wasp in sacs connected to her ovipositor, are injected into trees during oviposition (Talbot 1977). The spread of *Ophiostoma ulmi* Buisman, causing Dutch elm disease, is accomplished by transport of conidia of the fungus on exterior body surfaces of *Scolytus scolytis* Fabricius, a bark beetle (Webber and Gibbs 1989).

Insects may play an important role in the spread of *D. destructiva*. Earlier research with convergent lady beetles (CLBs), *Hippodamia convergens* Guerin-Meneville, has shown that the conidial matrix of *D. destructiva* makes possible the transport of viable conidia on the exterior surface of an insect. Conidia carried exteriorly on beetles were found to remain viable for as many as 16 days under laboratory conditions (Colby 1993,

Colby et al.1995, 1996) and in the field in shade (Holt 1995). However, beetles in sunlight carried viable conidia for only 1 day (Holt 1995). Lady beetles infested with conidia of *D. destructiva* were able to inoculate healthy dogwood foliage in a greenhouse inoculation chamber (Colby 1993).

Many species of arthropods have been collected from healthy and diseased dogwoods, representing several insect orders (Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, and Lepidoptera). Insect densities were greatest when the incidence of *D. destructiva* was at its highest levels (Neitch 1995). The frequency of arthropods infested with conidia of *D. destructiva* throughout the season followed changes in the rate of disease of dogwood anthracnose. Approximately 7% of insects collected from diseased dogwoods were carrying viable conidia on their bodies (Holt 1995).

Some fungi are capable of passing intact through the digestive system of insects. Conidia of *Botrytis cinerea* Pers. Fr. have been found on body surfaces and in the feces of moths feeding on infected fruit (Duffy and Gardner 1994). Conidia of *Verticillium albo-atrum* Reinke and Berthold, can be vectored externally on insects and in their feces [where conidia can overwinter (Harper et. al. 1988)] and are capable of causing new infections (Huang and Harper 1985). Migratory locusts, *Locusta migratoria* L. (which can cover long distances), feeding on diseased plants, ingested and discharged conidia of *Colletotrichum graminicola* Sesati that were capable of causing new infections. Other fungi, such as *Mucor, Aspergillus, and Penicillium* also were recovered from locust feces, indicating that fungal spores may be relatively unaffected by insect gut passage (Hasan 1982). Viable conidia of *D. destructiva* were found in the alimentary canals of CLBs

exposed to sporulating fruiting bodies of the fungus (Colby 1993, Colby et al. 1995, 1996). It is not known if ingested conidia can pass through the alimentary canal, be discharged in viable condition in the frass, and initiate new infections on healthy dogwood tissue. The objectives of this study, using the CLB as a model insect, were to: 1) develop a nonlethal method of surface-disinfesting adult CLBs, 2) determine if CLBs can discharge viable conidia of *D. destructiva* in frass, and if so, for how long, 3) quantify the amount of conidia in frass, and 4) determine if the CLBs, exposed to conidia of *D. destructiva* and then surface-disinfested, can initiate infection of healthy dogwood leaves in the greenhouse, by discharging viable conidia of *D. destructiva* in frass.

CHAPTER II

EXAMINATIONS OF CONVERGENT LADY BEETLE FRASS FOR PRESENCE AND VIABILITY OF CONIDIA OF *Discula destructiva.*

INTRODUCTION

Since dogwood anthracnose, caused by *Discula destructiva* Redlin (Hibben and Daughtrey 1988, Redlin 1991), was first observed in southeastern New York in 1978 (Pirone 1980), epidemics of this disease have spread throughout much of the eastern range of flowering dogwood, *Cornus florida* L. (Anderson et al. 1991). Little is known of the mechanisms by which this pathogen is disseminated. Rain splash and wind have been suggested as significant means of short distance dispersal of conidia of *D. destructiva* but do not fully account for the rapid spread of dogwood anthracnose (Daughtrey et al. 1996, Daughtrey and Hibben 1994). The fungus has been isolated from dogwood fruit and seeds, making birds and mammals potential carriers of conidia over long distances (Britton et al. 1993).

Insects also may be important in disseminating conidia of *D. destructiva*. As a model insect, convergent lady beetles (CLBs), *Hippodamia convergens* Guerin-Meneville, have been used to demonstrate that insects can become infested with conidia from conidiomata of *D. destructiva*, and that conidia may remain viable for as many as 16 days under laboratory conditions (Colby 1993, Colby et al. 1995, 1996). Lady beetles were capable of disseminating viable conidia from diseased to healthy foliage which subsequently

became infected and symptomatic (Colby 1993). Neitch (1995) found that the highest frequencies of insect visitation on dogwood occurred at the same time of year that epidemics of dogwood anthracnose were most rapidly expanding in Great Smoky Mountains National Park (GSMNP). Holt (1995) found that approximately 7% of insects collected from diseased dogwoods in GSMNP during the same time period carried viable conidia on their bodies. Viable conidia of *D. destructiva* also have been found in the alimentary canals (gut) of insects exposed to sporulating fruiting bodies of the fungus (Colby 1993, Colby et al. 1995, 1996). However , whether viable conidia can pass through the insect's gut and be discharged in frass is unknown.

The objectives of this study were to: 1) develop a nonlethal method of surface-disinfesting adult CLBs, 2) determine if CLBs can discharge viable conidia of *D. destructiva* in frass, and if so, for how long, and 3) quantify the amount of conidia in frass.

MATERIALS AND METHODS

Convergent lady beetles were selected as a model insect for these experiments because of their year-round availability (Rincon Vitova Insectaries, Oak View, CA), easy maintenance in the laboratory, and previous research using this insect in fungal dissemination studies (Colby 1993, Colby et al. 1995, 1996, Holt 1995, Neitch 1995). Beetles were stored in plexiglass containers in an incubator at 10°C with a 12/12 h light/dark cycle. Honey was provided for food, and water was supplied via a cotton-stoppered flask. Before beetles were used in experiments, they were taken from storage and allowed to acclimate to room temperature for about 30 min.

Isolates of *D. destructiva* were obtained from diseased trees near Sewanee and

Winchester, TN. Cultures were maintained in petri dishes on potato dextrose agar (PDA) amended with chlortetracycline hydrochloride and streptomycin sulfate, each at 25 mg/l (PDA+). To produce sporulating cultures used to infest CLBs, autoclaved leaves of *C. florida* were placed on the PDA+ (PDA+Cf) surface and then inoculated with a conidial suspension of *D. destructiva*. Cultures were incubated at 22°C with a 12/12 h light/dark cycle, and they usually sporulated in 10 days to 4 weeks.

Surface-disinfestation. CLBs were surface-disinfested to ensure that their frass would not be contaminated by conidia of *D. destructiva* from the external surface of CLBs. Adult CLBs were placed on a sporulating culture (20/dish, PDA+Cf) of *D. destructiva* (Sewanee isolate) for 1 h (infested CLBs). Beetles were then submerged and gently stirred with a stir bar in a solution of 0.525% sodium hypochlorite and 5% ethanol (10 ml/CLB) for either 0 s (control), 15 s, 30 s, 45 s, 60 s, 2 min or 5 min (20 CLBs/disinfestation period). The solution was then poured through a sterile screen that retained the beetles. An equal volume of sterile water was added and the beetles were submerged and gently stirred for 15 s. This rinse water was poured into a sterile container, and the beetles were retained by a sterile screen and placed in clean petri dishes (1 CLB/dish). Beetle mortality resulting from the disinfestation was recorded. Ten percent of the rinse water from each disinfestation period was placed on petri dishes of PDA in 1 ml aliquots (rinse water PDA plates), incubated for 10 days at room temperature (22°C), and observed for growth of *D. destructiva* (this step was included in all subsequent disinfestations). The longest disinfestation period resulting in ≤50% CLB mortality and the absence of growth of *D. destructiva* on the rinse water PDA plates was

determined and used as the standard disinfestation period for all subsequent experiments.

Conidial viability/time trial. Two groups of 30 beetles each were placed on sporulating cultures (15/dish, PDA+Cf) of *D. destructiva* (Winchester isolate) for 1 h. Beetles were then surface-disinfested in a solution of 0.525% sodium hypochlorite and 5% ethanol for 5 min, rinsed in sterile water for 15 s, and placed on clean petri dishes as described above.

After disinfestation, 17 survivors (43% mortality) of one group were provided tobacco aphids, *Myzus nicotianae* Blackman, as food for the duration of the experiment. A supply of tobacco aphids was maintained on tobacco plants, *Nicotiana tabacum* L., in the greenhouse and outdoors in insect-proof cages. Twenty-four survivors (20% mortality) of the other group were provided pieces of *Malus domestica* 'Red Delicious' Borkh., as food (0.125 cm^3/CLB). Beetles were transferred to clean petri dishes after 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, 72, 96, and 120 h, when the beetles were discarded. Frass pellets were collected in each interval. Each frass pellet was moistened, teased apart, and placed in 1 ml of sterile water in a 1.5 ml micro-centrifuge tube (Elkay Products, Shrewsbury, MA). Each pellet was allowed to soak overnight (16 to 24 h) and was then shaken on a vortex machine (Barnstead/Thermolyne, Dubuque, Iowa) for 2 min. The dissolved pellet was spread evenly on a petri dish of PDA+ and incubated at 22°C.

Controls included 75 beetles placed on uninoculated PDA+Cf (15/dish) for 1 h, and then surface-disinfested and rinsed as described above. Of 58 survivors after disinfestation (23% mortality), 50 were maintained on apple and used as controls for the conidial viability/time trial and hemacytometer conidial count experiments. The remaining eight

survivors were maintained on aphids after disinfestation and used as controls only for the conidial viability/time trial experiments. Frass pellets were dissolved and cultured as described above.

Any *Discula*-like colonies from the frass pellets were subcultured as necessary to remove contaminants. Transfers of *Discula*-like cultures were made to petri dishes of PDA+Cf and incubated at 22°C until sporulation. Colony, conidial, and fruiting body characteristics of these cultures were compared with those of *D. destructiva* to confirm the presence of viable conidia of *D. destructiva* in the frass. The percentage of frass pellets from which *D. destructiva* was isolated in each time interval and the theoretical binomial standard deviations were calculated (Ott 1993).

The length of time each CLB could carry viable conidia of *D. destructiva* internally was determined by the length of time viable conidia were discharged in frass. The percentage of CLBs carrying viable conidia at each time interval was calculated and described by a three parameter, single exponential decay equation (SAS Institute Inc., Cary, NC).

Hemacytometer conidial counts. Seventy-five CLBs were infested with conidia of *D. destructiva* and then surface-disinfested as described above. Fifty-six survivors (25% mortality) were transferred to clean petri dishes (1 CLB/dish) after 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, 72, 96, and 120 h. Beetles were maintained after disinfestation on apple. Frass pellets were collected after each interval and dissolved individually in 1 ml of water (as above). Samples were examined at 400x on a hemacytometer slide. Four hemacytometer readings were taken for each dissolved frass pellet, and estimates of the number of conidia/frass pellet were determined. The mean number of *Discula*-like

conidia/pellet and the standard error of the mean were calculated for each time interval. Frass pellets were collected from 63 (50 from conidial viability/time trial experiment plus 13 additional) CLBs placed on uninoculated PDA+Cf for 1 h and then surface-disinfested and rinsed. These CLBs served as controls for the hemacytometer experiments.

Scanning electron microscopy. Twenty-five CLBs were infested with conidia of *D. destructiva,* surface-disinfested, and rinsed as above. Seventeen survivors (32% mortality) were transferred to clean petri dishes every 2 h for 12 h. For controls, 15 CLBs were place on uninoculated PDA+Cf for 1 h, surface-disinfested, and rinsed as above. Thirteen survivors (13% mortality) were transferred to clean petri dishes every 2 h for 12 h. Three frass pellets from each interval (from CLBs exposed to *D. destructiva* and from controls) were moistened and teased apart on 12mm circular glass coverslips. These were air dried under a laminar flow hood for 2 h, and fixed for 1-2 h in 3% glutaraldehyde in 0.05M KH_2PO_4 buffer (pH 6.8). The samples were then rinsed in 3 successions of 0.05M KH_2PO_4 buffer (2 min/rinse) and transferred through a 30%, 50%, 70%, and 100% ethanol dehydration series (5 min/solution). Samples were critical point dried and sputter coated with gold for scanning electron microscopy. Micrographs were taken of frass from each interval (2, 4, 6, 8, 10, and 12 h) from CLBs exposed to *D. destructiva* and from controls at 600x magnification.

Refrigerated frass. A test was conducted to determine if conidia of *D. destructiva* could survive for long periods in insect frass. Extra frass pellets from the scanning electron microscopy experiment collected from CLBs exposed to *D. destructiva* were placed in petri dishes and refrigerated dry at 10°C for 4 months. After 4 months, pellets

were placed directly on PDA+ and incubated at 22°C. Mycelia of resulting *Discula*-like colonies were transferred to PDA+Cf until sporulation. Colony, conidial, and fruiting body characteristics from sporulating PDA+Cf cultures were compared with those of *D. destructiva*.

RESULTS

Surface-disinfestation. Mortality of CLBs caused by the disinfestation increased as disinfestation time increased. Mortality of the 0 s, 15 s, 45 s, and 60 s disinfestations was 0%. Mortality was 10% with the 30 s disinfestation, 15% with the 2 min disinfestation, and 50% with 5 min disinfestation.

Percent contamination of the rinse water plates decreased as disinfestation time increased. Of the 20 PDA rinse water plates for each duration, 100% of the 0 s disinfestation (control) rinse water plates showed contamination (bacterial and fungal growth) in 2 days. After 5 days, 35% of the 15 s disinfestation and 5% of the 30 s disinfestation rinse water plates showed fungal contamination (*Penicillium* spp. and *Aspergillus* spp.). All 45 s, 60 s, 2 min, and 5 min disinfestation rinse water plates showed 0% contamination. Only the control rinse water plates showed colonies with *Discula*-like growth. These *Discula*-like colonies were cultured on PDA+Cf until sporulation. Colony, conidial, and fruiting body characteristics were compared with those of *D. destructiva*, and *D. destructiva* was confirmed on 19 of the 20 control rinse water plates. The failure to isolate *D. destructiva* from all control plates was likely due to the difficulty of separating *D. destructiva* from the abundance of other contaminants, most of which grew faster than *D. destructiva*.

The 5 min disinfestation was used in all subsequent experiments because it provided greater assurance that exterior beetle surfaces were free of viable conidia of *D. destructiva*, while keeping beetle mortality at or below 50%. Data from all subsequent experiments using this method of the 5 min disinfestation produced an overall mortality rate of 29%. No *Discula*-like colonies were seen on any of the 5 min disinfestation rinse water plates.

Conidial viability/time trial. *Discula destructiva* was isolated from frass pellets of 76% (31/41) of the CLBs exposed to *D. destructiva*, and from frass in every interval. A three parameter, single exponential decay equation was generated for each diet where Y = percent CLBs carrying viable conidia internally and X = the time interval (Figure 1). For the aphid diet, $Y = -0.0452 + 0.716e^{-0.0466X}$, $R^2 = 0.96$. For the apple diet, $Y = 0.0897 + 0.850e^{-0.0331X}$, $R^2 = 0.95$. The percentage of CLBs carrying viable conidia of *D. destructiva* during each interval fell more slowly during the first 12 h than the next 12 h for both diets, before flattening out (Figure 1). The slopes of the curves were similar, but there was a marked difference in the initial percentage of CLBs carrying conidia and in the overall length of time CLBs carried conidia on the different diets (Figure 1). The percentage of frass pellets from which *D. destructiva* was isolated in each collection interval was consistently higher for CLBs fed apple than for CLBs fed aphids, with the exception of the 8-10 h interval (Table 1). No *Discula*-like colonies were isolated from any of the 454 frass pellets from the 58 control CLBs. *Discula destructiva* was isolated from 143 of 555 (26%) frass pellets collected from 41 CLBs exposed to *D. destructiva.*

Of the CLBs fed aphids, *D. destructiva* was isolated from frass of all intervals into the

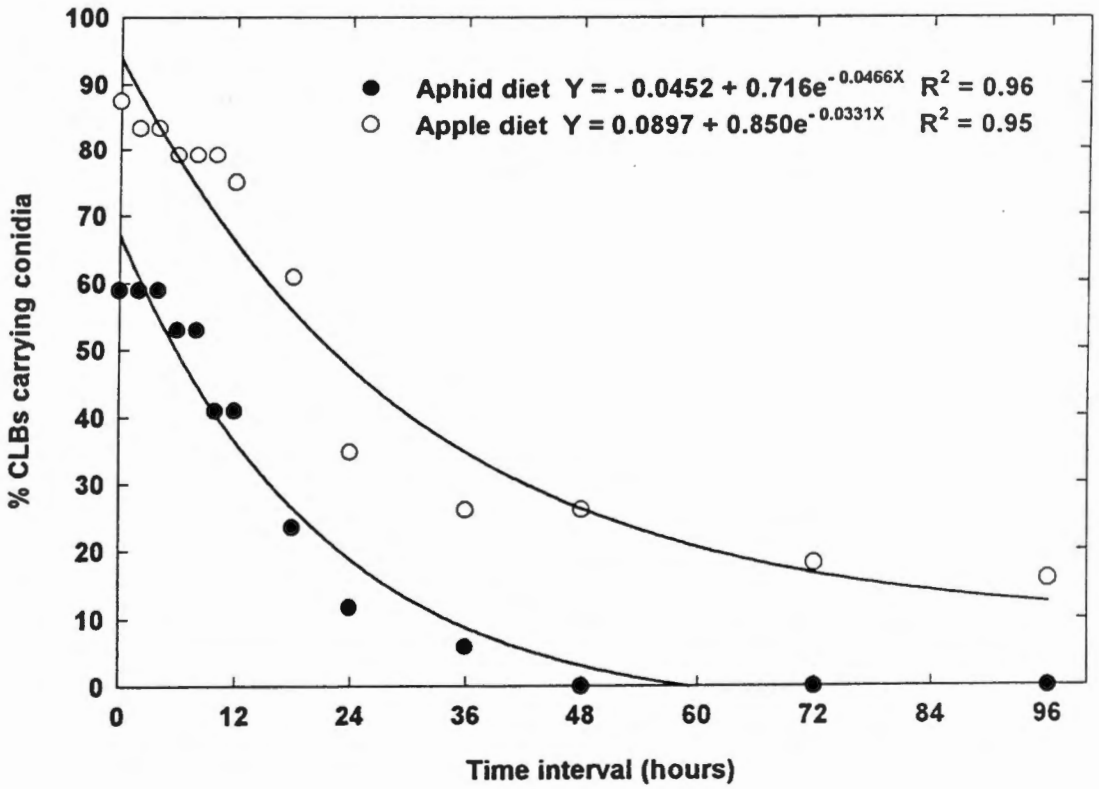


Fig. 1. Percentage of convergent lady beetles (CLBs) carrying viable conidia at specified time intervals after exposure to conidiomata of *Discula destructiva* and subsequent surface disinfestation.

Table 1. Percentage of frass pellets discharged from which *Discula destructiva* was isolated in each collection time for the conidial viability and time trial.

Time	% Frass pellets from which *Discula destructiva* was isolated	
	Aphid Diet	Apple Diet
2 h	20 (1/5)[a] ± 18[b]	100 (8/8) ± 0
4 h	43 (6/14) ± 13	75 (12/16) ± 11
6 h	50 (5/10) ± 16	82 (9/11) ± 12
8 h	50 (5/10) ± 16	56 (5/9) ± 17
10 h	50 (4/8) ± 18	40 (2/5) ± 22
12 h	50 (5/10) ± 16	67 (2/3) ± 27
18 h	21 (4/19) ± 9	65 (15/23) ± 10
24 h	24 (4/17) ± 10	59 (15/22) ± 10
36 h	10 (2/20) ± 7	38 (11/29) ± 9
48 h	6 (1/16) ± 6	28 (7/25) ± 9
72 h	0 (0/50)	20 (9/45) ± 6
96 h	0 (0/48)	21 (8/38) ± 7
120 h	0 (0/53)	7 (3/41) ± 4

[a] Numbers in parentheses refer to number of *Discula*-infested frass pellets/number of frass pellets discharged.

[b] Theoretical binomial standard deviation (Ott 1993).

36-48 h interval and from 59% (10/17) of the CLBs (Table 1). *Discula destructiva* was isolated from 37 of 280 (13%) pellets collected. More than 70% (26/37) of the pellets from which *D. destructiva* was isolated were discharged in the first 12 h. Ninety-two percent and more than 97% of the pellets from which *D. destructiva* was isolated had been discharged after 24 and 36 h, respectively.

Of the CLBs fed apple, *D. destructiva* was isolated from frass of all intervals and from 87.5% (21/24) of the CLBs (Table 1). *Discula destructiva* was isolated from 106 of 275 (39%) pellets collected. Only 36% (38/106) of the pellets from which *D. destructiva* was isolated were discharged in the first 12 h. Sixty-four percent, 81%, 90%, and 97% of the pellets from which *D. destructiva* was isolated had been discharged after 24, 48, 72, and 96 h, respectively.

Hemacytometer conidial counts. No *Discula*-like conidia were seen in any of the 426 frass pellets collected from 63 CLBs used as controls. The number of *Discula*-like conidia/pellet in frass of CLBs exposed to *D. destructiva* varied greatly within and between time intervals (Table 2). The percentage of frass pellets with *Discula*-like conidia fell most rapidly in the first 12 h. Mean number of *Discula*-like conidia/pellet fell gradually for the first 8 h and rose sharply in pellets collected at 10 and 24 h before falling again. *Discula*-like conidia were seen in 35% (256/724) of all frass pellets collected. The most conidia discharged by any one CLB was 1.69×10^7 conidia in 21 frass pellets within a 96 h period.

More than 76% of the *Discula*-like conidia counted were from frass pellets discharged within the first 24 h (75% of these conidia were discharged in the first 12 h). More than

Table 2. Hemacytometer conidial counts from frass of convergent lady beetles (CLBs) collected at specific times after exposure to *Discula destructiva.*

Time	No. CLBs*	% Frass pellets with *Discula*-like conidia	No. *Discula*-like conidia/pellet		
			Max	Mean	Std. Error
2 h	56	96 (22/23)[+]	1.68×10^6	5.63×10^5	$\pm 1.04 \times 10^5$
4 h	56	82 (27/33)	2.01×10^6	4.85×10^5	$\pm 1.08 \times 10^5$
6 h	56	82 (28/34)	1.59×10^6	4.17×10^5	$\pm 8.73 \times 10^4$
8 h	56	61 (17/28)	1.36×10^6	4.01×10^5	$\pm 1.07 \times 10^5$
10 h	56	71 (12/17)	2.29×10^6	9.18×10^5	$\pm 2.51 \times 10^5$
12 h	56	58 (11/19)	2.18×10^6	6.08×10^5	$\pm 2.13 \times 10^5$
18 h	56	48 (19/40)	1.47×10^6	5.05×10^5	$\pm 1.10 \times 10^5$
24 h	55	42 (21/50)	2.00×10^6	7.92×10^5	$\pm 1.42 \times 10^5$
36 h	55	40 (38/94)	3.21×10^6	4.67×10^5	$\pm 1.16 \times 10^5$
48 h	55	29 (19/65)	7.68×10^5	1.70×10^5	$\pm 5.16 \times 10^4$
72 h	55	17 (23/132)	7.55×10^5	1.70×10^5	$\pm 4.80 \times 10^4$
96 h	53	15 (14/95)	2.35×10^5	4.82×10^4	$\pm 1.74 \times 10^4$
120 h	49	5 (5/94)	1.25×10^4	6.00×10^3	$\pm 1.70 \times 10^3$

Means were based on the total number of conidia counted/number of pellets with *Discula*-like conidia. Minimum number of *Discula*-like conidia/pellet was 0 for all times. *Reductions in numbers of CLBs are due to mortality during experiment. [+]Numbers in parentheses refer to the number of *Discula*-infested pellets/number of frass pellets discharged.

95% and 99% of the conidia counted were discharged by the end of 48 and 72 h, respectively. Four CLBs were still discharging frass with *Discula*-like conidia into the 96-120 h period. *Discula*-like colonies were isolated from frass of three of these CLBs and from frass of a fifth CLB discharged in the 96-120 h interval in which no *Discula*-like conidia were seen on the hemacytometer. An estimated total of 1.09×10^8 *Discula*-like conidia had been discharged in the frass of 56 CLBs over a 120 h period.

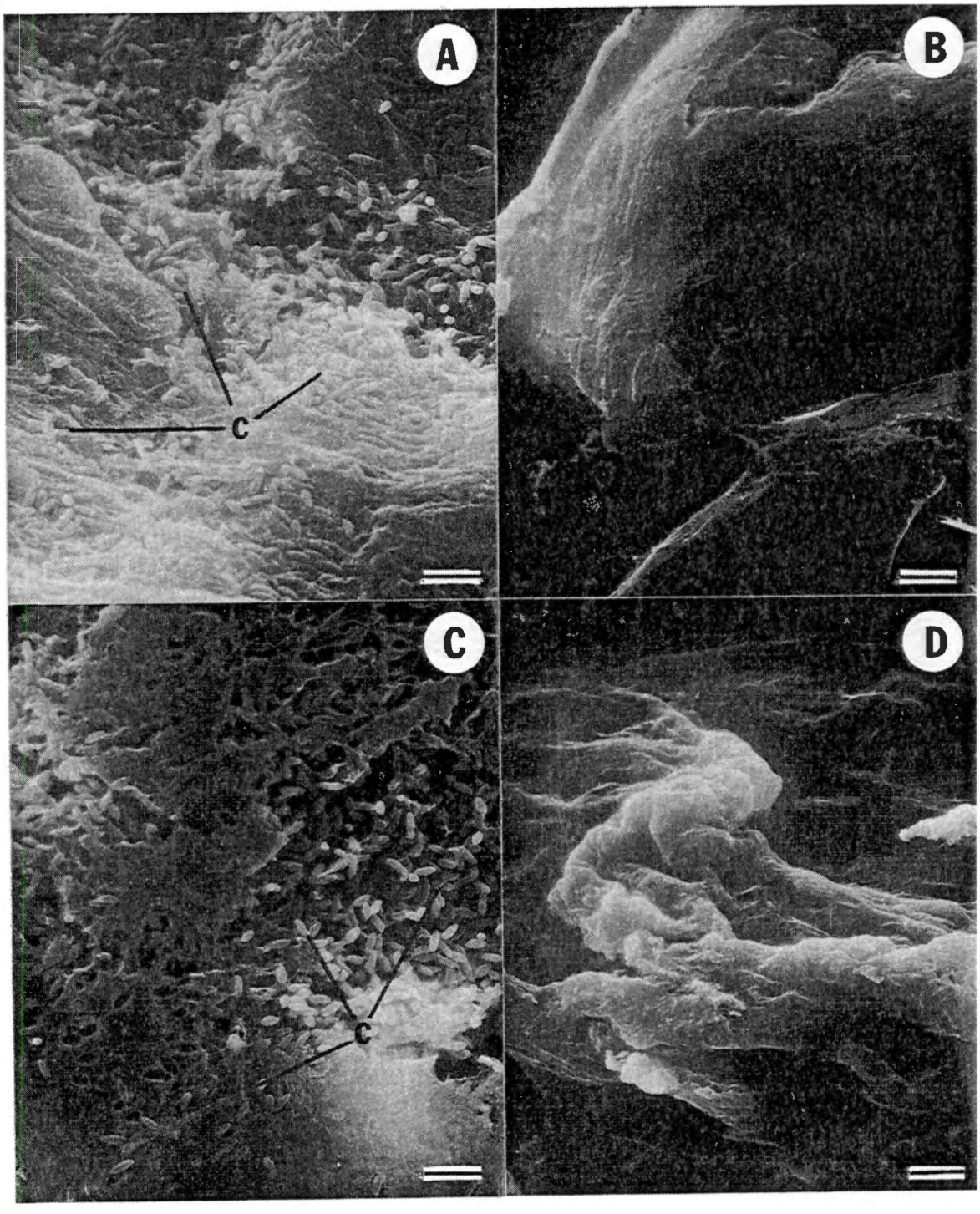
Scanning electron microscopy. Large numbers of *Discula*-like conidia were seen in frass collected in all 2 h intervals, up to 12 h, from CLBs exposed to *D. destructiva*. No *Discula*-like conidia were seen in frass of any interval from CLBs not exposed to *D. destructiva* (Figure 2).

Refrigerated frass. Frass pellets collected from CLBs exposed to *D. destructiva* and refrigerated for 4 months at 10°C yielded colonies of *D. destructiva*. *Discula destructiva* was isolated from frass collected 4, 6, 8, 10, and 12 h after disinfestation.

DISCUSSION

Surface-disinfestation. The 5 min Clorox disinfestation was used to ensure that any conidia of *D. destructiva* recovered from frass had passed through the insect's digestive system and was not from the exterior surface of the insect. Other studies testing for the presence of fungal propagules in insect frass have used a variety of materials and methods to surface-disinfest insects. Schroeder and Snow (1982) refrigerated weevils at 4°C (until weevil movement stopped) after exposure to fungal cultures. Weevils were then dipped in 4°C solutions of sodium hypochlorite for 45 sec and 95% ethanol for 5 sec. Some studies used only a sterile water rinse (Hasan 1982) of infested insects before collections of frass.

Figure 2. Scanning electron micrographs of convergent lady beetle (CLB) frass at 600x (c = conidia; bar = 25μm). (A) Frass from 0-2 h interval after exposure to conidia of *Discula destructiva* and surface disinfestation. (B) Frass of CLB without prior exposure to conidia of *D. destructiva*, 0-2 h after surface disinfestation (control). (C) Frass from 10-12 h interval after exposure to conidia of *D. destructiva* and surface disinfestation. (D) Frass of CLB without prior exposure to conidia of *D. destructiva*, 10-12 h after surface disinfestation (control).


A
C
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D

Fernaud and LeMenn (1989) examining frass of the grape berry moth, *Lobesia botrana* Denis and Schiffermueller, for conidia of *Botrytis cinerea* Pers. Fr., sterilized the discharged frass pellets in calcium hypochlorite solution for 1 min, and then rinsed the pellets in sterile distilled water. Disinfesting the pellets would have been difficult in this experiment due to the small size of the CLB pellets and increasing solubility of the pellets with increasing concentrations of conidia of *D. destructiva*.

The 5 min disinfestation procedure was effective for surface sterilizing live CLBs. The variation in each experiment in mortality of CLBs due to disinfestation ranged from 7 to 50%, and likely reflects variation in the hardiness of individual beetles in each disinfestation trial. No *D. destructiva* was isolated from any PDA plates inoculated with CLB rinse water from the 5 min disinfestation. A small percentage of these plates, 9 of 414 (2.2%), did show growth of bacteria and other fungi. Whether this contamination actually came from the rinse water or was already present when plates were inoculated is not known. Mites were found on 5 of the 9 contaminated plates and could have introduced fungi and bacteria onto these plates. In addition, some of the PDA plates were prepared the night before use, which may not have allowed enough time for accidental contamination during preparation of the plates to manifest itself. Therefore, some of the plates may have been contaminated before inoculation with CLB rinse water.

Conidial viability/time trial. CLBs generally did not resume feeding until 12-24 h after exposure to and ingestion of conidia of *D. destructiva*. Differences in diet would therefore not explain the marked differences in: 1) the percentages of frass pellets containing viable conidia during the first 6 h (Table 1) and 2) the initial percentages of

CLBs carrying conidia (Figure 1). However, differences in diet could have contributed to the differences in the overall length of time *D. destructiva* was recovered from frass. Other contaminants were more abundant in cultures from frass of CLBs on aphids than on apple. Therefore, the isolation of *D. destructiva* from CLBs on aphids can be made more difficult in later time intervals when the concentration of conidia of *D. destructiva* in frass is generally lower. The amount of inoculum ingested by individual CLBs also may affect the length of time conidia remain in the alimentary canal. In a study with *Verticillium albo-atrum* Reinke and Berthold, the time required for the pathogen to be eliminated from the digestive tracts of leaf-chewing insects was associated with the original level of inoculum individual insects ingested (Huang and Harper 1985).

Convergent lady beetles are predaceous, but will eat noninsect foods of plant origin when insects are not available (Hagen 1962). Apple was used as a substitute for aphids for several reasons: 1) the beetles were easily sustained on apple for the duration of the experiment, 2) there was less contamination from bacteria and other fungi in frass discharged by CLBs on the apple diet, making isolation of *D. destructiva* easier, and 3) frass from CLBs on apple contained less insoluble material and could be more thoroughly dissolved for the conidial counts.

Beetles allowed to feed on aphids for 24 and 48 h before exposure to sporulating cultures still ingested conidia of *D. destructiva*. Although detailed data from this experiment were not collected, these CLBs subsequently discharged viable conidia of *D. destructiva* in their frass for at least 12 to 24 h. However, the abundance of bacterial and fungal contaminants in their frass made isolation of *D. destructiva* difficult.

Hemacytometer conidial counts. Lack of food and water after removal from storage and before exposure to cultures may have influenced the quantity of conidia ingested and discharged in frass, making some of the conidial counts abnormally high. Convergent lady beetles seek moisture when coming out of storage (Hagen 1962) and slime droplets of conidia may have attracted them. Beetles were observed ingesting slime droplets of conidia and cleaning conidia-encrusted legs with their mouthparts. However, there was tremendous variation in the numbers of conidia discharged by different CLBs in each interval and over the duration of the experiment (10 CLBs had counts of 0 over the entire duration of the conidial count experiments).

Refrigerated frass. In studies with *V. albo-atrum*, length of time the pathogen remained viable in grasshopper feces increased with decreasing temperature (Harper et al. 1988). Conidia of *D. destructiva*, discharged in CLB frass and stored at 10°C remained viable for at least 4 months, and may serve as an overwintering source of inoculum.

Concluding remarks. Some of the most damaging fungal diseases of trees, such as Dutch elm disease, are known to be vectored by insects, particularly beetles (Webber and Gibbs 1989). Although in most cases, insects are not the primary means of dissemination of fungal propagules, interactions between insects and fungi often contribute to the spread and severity of plant disease. Larvae of the grape berry moth can increase the severity of fruit rot by *B. cinerea* (Fernaud and LeMenn 1992). *Verticillium albo-atrum* can be vectored externally and internally by leaf-chewing insects, where contaminated frass can serve as a source of inoculum for new infections on alfalfa, *Medicago sativa* L. (Harper et al. 1988, Huang and Harper 1985). Although used only as a model in these experiments,

even the CLB may play an important role (among other arthropods) in the distribution of *Cytospora* and wood rot fungi in stone fruit orchards (Helton et al. 1988a, 1988b).

Many studies on insect dissemination of fungal plant pathogens focus solely on external transmission and do not include an examination of insect frass for the presence of viable fungal propagules. Propagules of *Botrytis, Phytophthora, Verticillium, Colletotrichum* and *Verticicladiella* carried externally by insects are also found in insect frass (Duffy and Gardner 1994, Evans 1971, Harper et al. 1988, Hasan 1982, Huang and Harper 1985, Lewis and Alexander 1986). Although primarily wind disseminated, conidia of *B. cinerea* have been found on body surfaces and in the frass of grape berry moth larvae feeding on infected fruit (Duffy and Gardner 1994). Conidia of *B. cinerea* did not seem to be modified by passage through the digestive tract of the larvae, and dispersal of the pathogen via frass was demonstrated (Fernaud and LeMenn 1989). *Verticillium albo-atrum* survived passage through the digestive systems of *Melanoplus anguinipes* Fabricius (migratory grasshopper), *M. bivittatus* Say (two striped grasshopper), *Hypera postica* Gyllenhal (alfalfa weevil), and larvae of *Apantesis blakei* Grote (woolly bear). Frass from these insects containing *V. albo-atrum* were capable of causing new infections in alfalfa (Huang and Harper 1985). Examination of frass of several herbivorous invertebrates found on pods of cocoa, *Theobroma cacao* L., infected with *Phytophthora palmivora* (Butl.) Butl., revealed the presence of spores of *P. palmivora*. Pod inoculations from the frass of snails, millipedes, and caterpillars resulted in infection by *P. palmivora*. Although most sporangia in the frass of beetles showed evidence of damage by digestion and were of low infectivity, chlamydospores of *P. palmivora* pass through the invertebrate

digestive system unchanged (Evans 1971).

In this study, a number of fungi in addition to *D. destructiva* were isolated from frass of the CLB. Species of *Penicillium, Aspergillus, Botrytis, Trichoderma* and others that were not identified, were commonly encountered on CLB frass cultures. Hasan (1982) similarly noted the development of several common fungi such as *Mucor, Aspergillus*, and *Penicillium*, in addition to *Colletotrichum graminicola* Sesati on culture media inoculated by the gut contents of two species of Orthoptera. Conidia of *C. graminicola* showed no evidence of physical damage after passage through the alimentary canal and were capable of causing disease on maize after 56 h of gut passage. Fungal spores may be relatively unaffected by passage through the alimentary canal of an insect (Hasan 1982). Conidia able to survive in the alimentary canal may be protected from conditions of the external environment that destroy their viability. The sticky and fluid conidial matrix of *D. destructiva* may enhance the ingestion of conidia in the event of casual contact with insects and protect conidia (Louis and Cooke 1985, Nicholson and Moraes 1980) from the rigors of digestion.

Various insects are associated with both healthy and diseased dogwoods in the field. Some of these insects infested externally with conidia of *D. destructiva* (Holt 1995) also may ingest and discharge viable conidia in their frass. This study has shown that conidia of *D. destructiva* can survive in the alimentary canal of the CLB for up to 96 h and be discharged in viable condition. The discharge of insect frass with viable conidia of *D. destructiva* onto susceptible dogwood leaf surfaces may cause new infections and enhance the spread of dogwood anthracnose in the field.

CHAPTER III

INFECTION OF *Cornus florida* IN A GREENHOUSE INOCULATION CHAMBER BY CONVERGENT LADY BEETLES EXTERNALLY AND INTERNALLY INFESTED WITH CONIDIA OF *Discula destructiva*

INTRODUCTION

Insects contribute to the spread and severity of many plant pathogens and may play a role in disseminating conidia of *Discula destructiva* Redlin. Convergent lady beetles (CLBs), *Hippodamia convergens* Guerin-Meneville, used as a model insect, are capable of disseminating viable conidia of *D. destructiva* from diseased to healthy foliage of flowering dogwood, *Cornus florida* L., in the greenhouse, leading to symptoms of dogwood anthracnose (Colby 1993). Viable conidia of *D. destructiva* also have been found in the alimentary canals of CLBs exposed to sporulating fruiting bodies of the fungus (Colby 1993, Colby et al. 1995, 1996). However, it is not known whether conidia surviving passage through the alimentary canal and discharged in frass onto susceptible dogwood leaf surfaces can incite new infections. Therefore, the objective of this study was to determine if CLBs exposed to conidia of *D. destructiva* and then surface-disinfested, can initiate infection of healthy dogwood leaves by discharging viable conidia in frass.

MATERIALS AND METHODS

Convergent lady beetles, selected as a model insect for these experiments, were acquired from Rincon-Vitova Insectaries, Oak View, CA. Beetles were stored in plexiglass containers in an incubator at 10°C with a 12/12 h light/dark cycle. Honey was provided for food, and water was supplied via a cotton-stoppered flask. Before their use in these experiments, CLBs were taken from storage and allowed to acclimate to room temperature for about 30 min.

Isolates of *D. destructiva* were obtained from trees symptomatic for dogwood anthracnose near Winchester, Signal Mountain, and Oak Ridge, TN. Cultures were maintained in petri dishes on potato dextrose agar (PDA) amended with chlortetracycline hydrochloride and streptomycin sulfate, each at 25 mg/l (PDA+). To produce sporulating cultures used to infest CLBs, autoclaved leaves of *C. florida* were placed on the PDA+ surface (PDA+Cf) and then inoculated with either mycelia or a conidial suspension of *D. destructiva*. Cultures were incubated at 22°C with a 12/12 h light/dark cycle, and they usually sporulated in 10 days to 4 weeks.

Experiments were conducted in a greenhouse inoculation chamber where environmental conditions conducive to infection of dogwood leaves by *D. destructiva* could be maintained for 4 weeks. The chamber consisted of a wooden frame 444 cm long x 150 cm wide x 136 cm high, covered with two layers of clear plastic separated by 3.8 cm of air space. Three hinged doors down one length of the chamber provided access to the interior. The chamber was equipped with an air conditioner to control maximum temperature, a humidifier to help maintain 100% humidity, and a shade cloth to cover the

chamber. The experiment was conducted in the spring of 1997 and repeated with variations in the spring of 1998.

Spring 1997. Ten potted *C. florida* 'Cherokee Brave' trees, 90 to 120 cm tall, were allowed to break dormancy in an outdoor nursery compound. The trees were then placed in the greenhouse in the inoculation chamber which was covered with 90% shade cloth. Five trees were inoculated with the *D. destructiva* isolate from Signal Mountain, TN, and five trees were inoculated with the *D. destructiva* isolate from Winchester, TN. Each tree received one replication of four different inoculation treatments on each of four branches. Treatments were as follows: no CLBs (NB), 5 CLBs that had not been exposed to fungal cultures (non-infested beetles or NIB), 5 CLBs exposed to sporulating cultures of *D. destructiva* for 1 h (externally infested beetles or EIB), and 5 CLBs that had been exposed to sporulating cultures of *D. destructiva* for 1 h and then surface-disinfested (infested/disinfested beetles or IDB). Beetles for IDB treatments were surface-disinfested by being submerged and stirred in a solution of 0.525% sodium hypochlorite and 5% ethanol (5 ml/CLB) for 5 min. The solution was then poured through a sterile screen that retained the CLBs. CLBs were rinsed by being submerged and stirred in an equal volume of sterile water for 15 s. The rinse water was poured through a sterile screen that retained the CLBs. Beetle mortality from the disinfestation was recorded.

Each replication of a treatment consisted of about ten leaves/replication. All treatments were covered with clear plastic bags sealed with a twist tie. CLBs remained on treatments for 24 h (dead ones were replaced as needed). After 24 h CLBs were discarded and bags were resealed for 4 weeks. Humidity was maintained at 100%, and temperatures ranged

from 11 to 25°C. After 4 weeks, leaves were harvested and placed in moist chambers consisting of a petri dish with moistened filter paper and sealed with parafilm. Leaves were examined for *Discula*-like acervuli after 1 and 2 weeks. Conidia from sporulating fruiting bodies were placed on petri dishes of PDA+ and incubated at 22°C. Mycelia from any *Discula*-like fungal colonies were subcultured to remove contaminants. Transfers of purified *Discula*-like cultures were made to petri dishes of PDA+Cf and incubated at 22°C until sporulation. Colony, conidial, and fruiting body characteristics of these cultures were compared with those of *D. destructiva* used to originally infest the CLBs to confirm the isolation of *D. destructiva*. The percentage of treatments infected with *D. destructiva* was determined for each treatment.

Spring 1998. Eighteen potted *C. florida* 'Cherokee Brave', 90 to 120 cm tall, were moved from an unheated plastic covered greenhouse into a heated greenhouse to break dormancy. After dormancy was broken, trees were placed in the inoculation chamber described above which was covered with 98% shade cloth. Isolates of *D. destructiva* from Winchester, TN and Oak Ridge, TN were used. Each tree received three replications of one treatment on three separate branches, and each treatment (three trees/treatment) was replicated nine times. Each replication of a treatment consisted of about 12.5 leaves/replication.

Treatment conditions were the same as for spring 1997 with one exception. Surface-disinfestation of CLBs in IDB treatments was amended by using a cup in cup method. A clear plastic cup was punctured at the bottom with 20 holes (2 mm in diameter). This cup was placed inside a larger clear plastic cup without holes that was filled with the same

disinfesting solution as described above (10 ml/CLB). CLBs were added to the inner cup, submerged and stirred with a stir bar for 5 min. The inner cup was removed and the disinfesting solution allowed to drain while retaining the CLBs. The inner cup was transferred to another larger cup filled with an equal volume of sterile rinse water. CLBs were submerged and stirred for 15 s. The inner cup was removed and the rinse water allowed to drain back into the outer cup while retaining the CLBs. Beetle mortality resulting from the disinfestation was recorded. One hundred percent of the rinse water from the disinfestation was spun in a centrifuge at 10,000 rpm for 15 min. The supernatant was poured off, and any sediment remaining was resuspended in sterile water and placed on petri dishes of PDA in 1 ml aliquots, incubated for 10 days at room temperature (22°C), and observed for growth of *D. destructiva*.

All treatments were covered with clear plastic bags for 4 weeks. Five CLBs were maintained on each beetle treatment for the first 24 h (dead ones were replaced as needed). After 24 h, CLBs were kept on treatments for another 9 days, but dead CLBs were not replaced. After 10 days, all remaining CLBs were removed from treatments and bags were resealed for the remainder of the 4 weeks. Humidity was maintained at 100% throughout the experiment, and temperatures ranged from 16 to 24°C. After 4 weeks, all treatment leaves were harvested, placed in moist chambers, and examined as above. Conidia from sporulating fruiting bodies were cultured and verified for *D. destructiva* as above. The percentage of treatments that became infected with *D. destructiva* was determined for each treatment. Statistical analysis was conducted using Chi-square and Fisher's exact test (SAS Institute Inc., Cary, NC), to determine if there were significant

differences between treatments in the percentage of treatments that became infected with *D. destructiva.*

RESULTS

Spring 1997. *Discula destructiva* was isolated from 60% (3/5) of EIB and IDB treatments using the Winchester isolate. *Discula destructiva* could not be isolated from any of the EIB and IDB treatments using the Signal Mountain isolate, or from any of the control treatments NB and NIB (Table 3). Beetle mortality from the disinfestation was 21% (13/63).

Spring 1998. *Discula destructiva* was isolated from 89% (8/9) of EIB treatments for both the Winchester and the Oak Ridge isolates. *Discula destructiva* was isolated from 56% (5/9) of IDB treatments for the Oak Ridge isolate and 11% (1/9) of IDB treatments for the Winchester isolate. *Discula destructiva* could not be isolated from any of the control treatments NB and NIB (Table 3). Beetle mortality from the disinfestation was 18% (36/200). *Discula destructiva* could not be isolated from any of the water used to rinse the CLBs after disinfestation. The percentage of treatments that became infected was significantly different between EIB and IDB treatments for the Winchester isolate ($df = 1$, $P < 0.01$), but not for the Oak Ridge isolate ($df = 1$, $P > 0.05$).

DISCUSSION

Spring 1997. No differences in the success of insect inoculation between CLBs infested with conidia externally (EIB treatments) and CLBs infested with conidia and then surface-disinfested (IDB treatments) were observed for either isolate. Differences, however, were noted between isolates. It is not known why no treatments became infected using

Table 3. Percentage of treatments (isolate/beetle treatment) infected with *Discula destructiva*.

Spring 1997

Isolate	Treatment	% Infected Treatments
Winchester, TN	No CLBs	0 (0/5)[a]
	Non-infested CLBs	0 (0/5)
	Externally Infested CLBs	60 (3/5)
	Infested/Disinfested CLBs	60 (3/5)
Signal Mountain, TN	No CLBs	0 (0/5)
	Non-infested CLBs	0 (0/5)
	Externally Infested CLBs	0 (0/5)
	Infested/Disinfested CLBs	0 (0/5)

Spring 1998

Isolate	Treatment	% Infected Treatments
Winchester, TN	No CLBs	0 (0/9)[a]
	Non-infested CLBs	0 (0/9)
	Externally Infested CLBs	89 (8/9)
	Infested/Disinfested CLBs	11 (1/9)
Oak Ridge, TN	No CLBs	0 (0/9)
	Non-infested CLBs	0 (0/9)
	Externally Infested CLBs	89 (8/9)
	Infested/Disinfested CLBs	56 (5/9)

[a] Number of replications that contained at least one leaf that became infected by *D. destructiva* /Total number of replications.

the Signal Mountain isolate. Conidia from the PDA+Cf cultures of the Signal Mountain isolate used to infest the CLBs were later tested for viability and produced typical cultures of *D. destructiva* on PDA+. It is possible that the isolate from Signal Mountain was less virulent than the isolate from Winchester. Another possibility is that conidia produced by the isolate from Signal Mountain may not survive conditions in the inoculation chamber. This isolate was replaced by the Oak Ridge isolate for the 1998 experiment.

Spring 1998. Inoculation with CLBs externally infested (EIB treatments) resulted in a greater percentage of infected treatments than inoculation with CLBs only carrying conidia internally (IDB treatments). In previous studies, CLBs externally infested also carried conidia internally and ingested conidia were viable and/or transported 95% and 100% of the time (Colby 1993, Colby et al. 1995, 1996). Therefore, the higher percentage of infected treatments with externally infested CLBs may be due to the combined effects of conidia deposited from external surfaces and conidia discharged in frass.

Differences in the percentage of infected treatments using externally infested CLBs (EIB treatments) occurred between years. In 1997, only 30% or 3 of 10 treatments (0 of 5 treatments using Signal Mountain isolate, and 3 of 5 treatments using the Winchester isolate) inoculated with externally infested CLBs became infected. In 1998, 89% or 16 of 18 treatments (8 of 9 treatments using Winchester isolate, and 8 of 9 treatments using Oak Ridge isolate) inoculated with externally infested CLBs became infected. In 1998, CLBs were left on treatments for up to 10 days as opposed to only 1 day in 1997, which likely

enhanced the probability of infection. Externally carried conidia can remain viable for as many as 16 days in the laboratory (Colby 1993, Colby et al. 1995, 1996) and in the shade in the field (Holt 1995). The mechanisms by which, and the extent to which, longer exposure (more than 24 h) of leaves to infested CLBs enhanced the probability of infection is unclear. The high humidity levels consistently maintained in the treatment bags may quickly reduce conidial viability and restrict the length of time that externally carried conidia are capable of resulting in infection (Roncadori 1993). In addition, the percentages of infected treatments from the 1998 experiment for externally infested CLBs (89% for both isolates), more closely agree with those of Colby (1993) for externally infested CLBs and nonwounded leaves (100% and 60%) in which infested CLBs were on treatments for only 24 h. Most successful inoculations probably occurred in the first 24 h.

The percentage of infected treatments with surface-disinfested CLBs was only slightly different between 1997 and 1998. The percentage of infected treatments from both isolates combined in 1997 was 30% or 3 of 10 treatments (0 of 5 treatments using the Signal Mountain isolate, and 3 of 5 treatments using the Winchester isolate) and in 1998 was 33% or 6 of 18 treatments (1 of 9 treatments using the Winchester isolate, and 5 of 9 treatments using the Oak Ridge isolate). Infection by conidia carried internally appears to be most effective in the first 24 h, as the percentage of infected treatments were only slightly different when CLBs were on treatments for 1 day (1997) or 10 days (1998). *Discula*-like acervuli were observed on a greater number of treatments, but an abundance of other fungal contaminants often made isolation of the fungus difficult or unsuccessful.

Other studies have shown that plant pathogenic fungi, surviving passage through the

alimentary canal of an insect are capable of initiating infections on susceptible plant tissue. Conidia of *Colletotrichum graminicola* (Sesati) G. W. Wilson recovered in frass of migratory locusts, *Locusta migratoria* L., and desert locusts, *Schistocerca gregaria* Forsk., were capable of causing disease symptoms on their host plants (Hasan 1982). Propagules of *Verticillium albo-atrum* Reinke and Berthold discharged in frass of migratory grasshoppers, *Melanoplus anguinipes* Fabricius, two striped grasshoppers, *M. bivittatus* Say, alfalfa weevils, *Hypera postica* Gyllenhal, and woolly bear larvae, *Apantesis blakei* Grote, were still capable of causing infections on alfalfa, *Medicago sativa* L. (Huang and Harper 1985).

These results suggest that conidia of *D. destructiva* discharged in frass of the CLB are viable and capable of causing new infections on susceptible dogwood leaf surfaces. In the greenhouse, insect-mediated inoculation of leaves with conidia of *D. destructiva* may be accomplished by conidia carried externally and/or internally, and without wounding the leaves. The role of insect-mediated inoculation of dogwoods with *D. destructiva* may be significant because various arthropods can carry viable conidia of *D. destructiva* on their bodies in the field (Holt 1995). Ingestion of conidia of *D. destructiva* by insects (through leg cleaning with mouthparts) and subsequent discharge of conidia in frass may enhance the spread of dogwood anthracnose.

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APPENDIX

INTRODUCTION

During this research, alternative methods were evaluated to isolate *Discula destructiva* Redlin, causal agent of dogwood anthracnose, from frass of the convergent lady beetle (CLB), *Hippodamia convergens* Guerin-Meneville. These experimental evaluations were not designed to be compared to previous experiments conducted in this manuscript. However, these evaluations are included to show that subtle variations in the experimental methods used may produce variations in the results, and may be of interest to future research. These results also provide further evidence for the survival of *D. destructiva* through the alimentary canal of an insect.

The materials and methods used in these evaluations to isolate *D. destructiva* from frass of the CLB are essentially the same as in chapter II for **surface-disinfestation** and **conidial viability/time trial** with one exception. Individual frass pellets collected from CLBs exposed to *D. destructiva* were placed directly on the surface of a PDA+ petri plate without prior dissolution in 1 ml of sterile water. The data gathered from this experiment from **surface-disinfestation** and **conidial viability/time** trial were analyzed and presented in the same manner as the data gathered in chapter II.

MATERIALS AND METHODS

Surface-disinfestation. Fifty adult CLBs were taken from cold storage, allowed to acclimate to room temperature for about 30 min and placed on sporulating cultures (10/dish, PDA+Cf) of *D. destructiva* (Winchester isolate) for 1 h. Beetles were then submerged and gently stirred with a stir bar in a solution of 0.525% sodium hypochlorite and 5% ethanol (10 ml/CLB) for 5 min. The solution was then poured through a sterile

screen that retained the CLBs. An equal volume of sterile water was added and the CLBs were submerged and gently stirred for 15 s. This rinse water was poured into a sterile container, and the CLBs were retained by a sterile screen and placed in clean petri dishes (1 CLB/dish). Beetle mortality from the disinfestation was recorded. Ten percent of the rinse water was placed on petri dishes of PDA in 1 ml aliquots (rinse water plates), incubated for 10 days at 22°C and observed for growth of *D. destructiva.*

Conidial viability/time trial. After disinfestation, 27 survivors were provided tobacco aphids as food for the duration of the experiment. Beetles were transferred to clean petri dishes after 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, 72, 96, and 120 h. Frass pellets were collected in each interval. Individual frass pellets were moistened in a drop of water and transferred directly to petri dishes of PDA+ and incubated at 22°C. Any *Discula*-like colonies from the frass pellets were subcultured as necessary to remove contaminants. Transfers of the purified colonies were made to petri dishes of PDA+Cf and incubated at 22°C until sporulation. Colony, conidial, and fruiting body characteristics of these cultures were compared with those of *D. destructiva* to confirm the presence of viable conidia of *D. destructiva* in the frass. The percentage of frass pellets from which *D. destructiva* was isolated in each time interval and the theoretical binomial standard deviations were calculated. The length of time each CLB could carry viable conidia of *D. destructiva* internally was determined by the length of time viable conidia were discharged in frass. The percentage of CLBs carrying viable conidia at each time interval was calculated and described by a three parameter, single exponential decay equation (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Surface-disinfestation. Mortality of the CLBs from the disinfestation was 46% (23/50). *Discula destructiva* could not be isolated from any of the CLB rinse water plates.

Conidial viability/time trial. *Discula destructiva* was isolated from 100% (27/27) of the CLBs and from all intervals into the 48-72 h interval (Table 4). *Discula destructiva* was isolated from 27% (66/246) of the pellets collected. More than 75% of the pellets from which *D. destructiva* was isolated (50/66) were discharged in the first 12 h, most of which (31/50) occurred between 2 and 6 h. Ninety-two percent and 98% of the pellets from which *D. destructiva* was isolated had been discharged after 24 and 48 h, respectively. A three parameter, single exponential decay equation was generated, $Y = 0.0155 + 1.055e^{-0.108X}$, $R^2 = 0.98$, where Y = percent CLBs carrying viable conidia internally and X = the time interval (Figure 3). The percentage of CLBs carrying conidia fell quickly to only 11% after 24 h, falling more rapidly in the first 12 h than in the second 12 h, before flattening out.

The results of this experiment have shown that conidia of *D. destructiva* can survive passage through the alimentary canal of the CLB and be discharged in viable condition in frass. The difference between this experiment and those of chapter II is the direct method of transferring frass to petri dishes of PDA+ . The direct method was a simple, reasonably effective method for the isolation of *D. destructiva* from CLB frass. The dissolution method of chapter II was preferred based on the assumption that by diluting the contents of a frass pellet and spreading the pellet over the entire surface of the PDA plate, one might more effectively recover *D. destructiva* from among faster growing contaminants.

Table 4. Percentage of frass pellets discharged from which *Discula destructiva* was isolated in each collection time.

Time Collected	% Frass pellets from which *Discula destructiva* was isolated
2 h	83 (5/6)[a] ± 15[b]
4 h	68 (17/25) ± 9
6 h	78 (14/18) ± 10
8 h	45 (5/11) ± 15
10 h	41 (7/17) ± 12
12 h	15 (2/13) ± 10
18 h	39 (7/18) ± 11
24 h	21 (4/19) ± 9
36 h	15 (3/20) ± 8
48 h	4 (1/24) ± 4
72 h	4 (1/24) ± 4
96 h	0 (0/25)
120 h	0 (0/25)

[a] Numbers in parentheses refer to number of *Discula*-infested frass pellets/number of frass pellets discharged.

[b] Theoretical binomial standard deviation (Ott 1993).

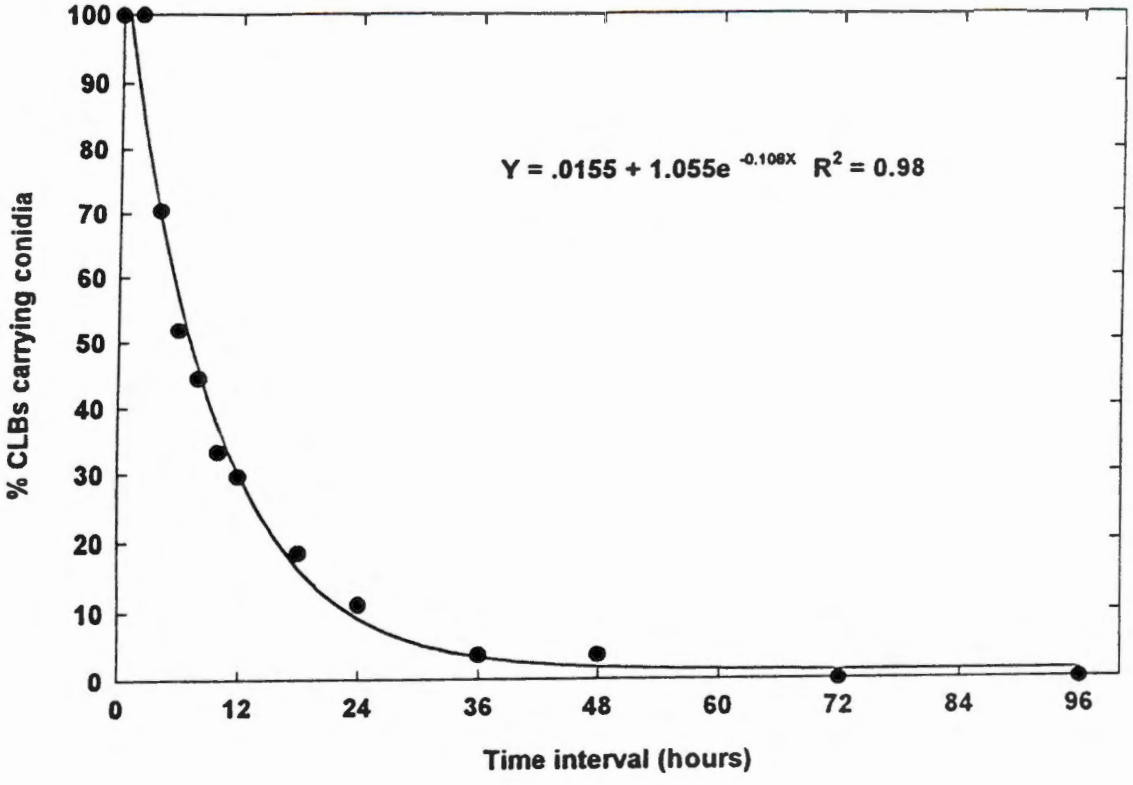

Fig. 3. Percentage of convergent lady beetles (CLBs) carrying viable conidia at specified time intervals after exposure to conidiomata of *Discula destructiva* and subsequent surface-disinfestation (direct plating method).

VITA

Bryan Hed was born in Chicago, Illinois, on October 15, 1957. Upon graduating from Niles West High School in August 1975, he attended the University of Illinois in Urbana, and received his B.S. in biology/ecology in 1979. After his marriage to Megan Collins in 1980, he had a son, Andrew, in 1985, and a daughter, Lara, in 1987. He remained in Urbana, Illinois until 1994. He and his family moved to Salt Lake City, Utah in 1994, and then to Menasha, Wisconsin in 1995. In 1996 he accepted a teaching assistantship at the University of Tennessee, Knoxville, and began work on a Masters degree in the department of entomology and plant pathology.

Bryan Hed is a member of the American Phytopathological Society, the American Association of Botanic Gardens and Arboreta, the Tennessee Entomological Society, and Gamma Sigma Delta.