Survival of *Conidia of Discula destructiva* in Frass of the Convergent Lady Beetle

Bryan E. Hed, Graduate Research Assistant, Mark T. Windham, Associate Professor, and Jerome F. Grant, Professor, Department of Entomology and Plant Pathology, University of Tennessee, Knoxville 37901-1071

ABSTRACT

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The survival of conidia of *Discula destructiva* in frass of convergent lady beetles (*Hippodamia convergens*) was investigated. *D. destructiva* was isolated from frass pellets of 76% of adult convergent lady beetles exposed to *D. destructiva* for 1 h. Of the beetles from which *D. destructiva* was initially isolated, more than 80, 32, 19, 13, and 12.5% retained viable conidia of *D. destructiva* internally for at least 12, 24, 48, 72, and 96 h, respectively. Numbers of conidia, estimated with a hemacytometer, ranged from 0 to 3.2×10^6 conidia per frass pellet.

Additional keywords: *Cornus florida*, dogwood anthracnose, flowering dogwood

Epidemics of dogwood anthracnose, caused by *Discula destructiva* Redlin (14,20), have occurred throughout much of the eastern range of flowering dogwood, *Cornus florida* L. (1). 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 they do not fully account for the rapid spread of dogwood anthracnose (6,7). The fungus has been isolated from dogwood fruits and seeds, making birds and mammals potential carriers of conidia over long distances (2).

Insects also may be important in disseminating conidia of *D. destructiva*. As a model insect, the convergent lady beetle, *Hippodamia convergens* Guérin-Ménéville (Coleoptera: Coccinellidae), has 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 (3–5). Lady beetles were capable of disseminating viable conidia from diseased to healthy foliage, which subsequently became infected and symptomatic (3). Neitch (18) 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. Holt (15) found that approximately 7% of insects collected from diseased dogwoods in the same park during the

same time period carried viable conidia on their bodies.

Many studies on insect dissemination of fungal plant pathogens have focused solely on external presence of pathogens and do not include an examination of insect frass for the presence of viable fungal propagules. Propagules of *Botrytis*, *Phytophthora*, *Verticillium*, *Colletotrichum*, and *Verticicladiella* spp. carried externally by insects are also found in insect frass (8,9,11,12,16,17). Survival of a pathogen in insect frass may offer an additional means of increasing the distribution of the pathogen and the spread of plant disease. In laboratory studies, viable conidia of *D. destructiva* have been found in the alimentary canals of convergent lady beetles exposed to sporulating fruiting bodies of the fungus (3–5). However, whether viable conidia of *D. destructiva* can pass through the insect's alimentary canal and be discharged in frass is unknown.

Convergent lady beetles were selected as a model insect for this experimental study because of their year-round commercial availability, easy maintenance in the laboratory, previous research using this insect in fungal dissemination studies (3–5), and their presence in forested and urban areas of the Southeast (10). The objectives of this study were to determine if convergent lady beetles can discharge viable conidia of *D. destructiva* in frass after ingesting conidia, and if so for how long, and to quantify the conidia in frass.

MATERIALS AND METHODS

Maintenance of insects and fungus.

Live beetles obtained from Rincon Vitova Insectaries, Oak View, California, were stored for up to 6 months before use in Plexiglas 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.

An isolate of *D. destructiva* was obtained from diseased trees near Winchester, Tennessee. Cultures were maintained in petri dishes on potato dextrose agar (PDA) amended with chlortetracycline hydrochloride and streptomycin sulfate, each at 25 mg/liter (PDA+). To produce sporulating cultures used to infest the beetles, autoclaved leaves of *C. florida* (Cf) 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.

Visual detection and quantification of conidia in frass. Seventy-five beetles were allowed to walk on the surface of sporulating cultures (15 per dish, PDA+Cf) of *D. destructiva* for 1 h to ingest conidia. For controls, 75 beetles were allowed to walk on uninoculated PDA+Cf (15 per dish) for 1 h. All beetles were then submerged and gently stirred with a stir bar in a solution of 0.5% sodium hypochlorite and 5% ethanol (10 ml per beetle) for 5 min. 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. The rinse water was poured out, and the surface-sterilized beetles were retained with a sterile screen and placed in clean petri dishes (1 beetle per dish). Survivors of the surface-sterilization, 56 (25% mortality) that had been exposed to *D. destructiva* and 63 (16% mortality) of the control group, were maintained after surface-sterilization on pieces of *Malus domestica* Borkh. 'Red Delicious' as food (0.125 cm³ per beetle). All beetles were transferred to clean petri dishes (one beetle per dish) with a fresh supply of food after 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, 72, 96, and 120 h, after which the beetles were discarded. All frass pellets were collected after each interval, and each frass pellet was moistened, teased apart, and placed in 1 ml of sterile water in a 1.5-ml microcentrifuge 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, IA) for 2 min. Samples were examined at $\times 400$ on a hemacytometer slide. Four hemacytometer readings were taken for each dissolved

Corresponding author: M. T. Windham
E-mail: mwindham@utk.edu

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frass pellet, and estimates of the number of conidia per frass pellet were determined. The mean number of *Discula*-like conidia per pellet and the standard error of the mean were calculated for each time interval.

Scanning electron microscopy was used to visually detect the presence of conidia in frass. Three frass pellets collected every 2 h for 12 h, from beetles exposed to *D. destructiva* and from controls, were moistened, teased apart, air-dried on 12-mm cover glasses, and fixed in 3% glutaraldehyde in 0.05 M KH_2PO_4 buffer (pH 6.8). After dehydration, samples were critical-point dried and sputter-coated with gold for scanning electron microscopy.

Viability of conidia in frass. Two groups of 30 beetles each were allowed to walk on sporulating cultures (15 per dish, PDA+Cf) of *D. destructiva* for 1 h. For controls, 75 beetles were allowed to walk on uninoculated PDA+Cf (15 per dish) for 1 h. All beetles were then surface-sterilized and rinsed as described above. Of the 60 beetles exposed to *D. destructiva*, 17 survivors of the surface-sterilization (43% mortality) of one group were provided tobacco aphids, *Myzus nicotianae* Blackman, as food for the duration of the experiment. Tobacco aphids were 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 maintained on apple. Of 58 survivors (23% mortality) of the control group, 50 were maintained on apple and eight were maintained on aphids. All beetles were transferred to clean petri dishes (1 beetle per dish) with a fresh supply of food after 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, 72, 96, and 120 h, after which the beetles were discarded. All frass pellets were collected after each interval, individually macerated as above, spread evenly on a petri dish of PDA+, and incubated at 22°C.

Discula-like colonies from the frass pellets were subcultured as necessary to remove contaminants. Transfers of these *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*. The percentage of frass pellets from which *D. destructiva* was isolated in each time interval and the theoretical binomial standard deviations were calculated (19).

The length of time each beetle could carry viable conidia of *D. destructiva* internally was determined by the length of time viable conidia were discharged in frass. The percentage of beetles carrying viable conidia at each time interval was calculated and described by a three-parameter, single exponential decay equation (SAS/STAT User's Guide: Statistics, Ver. 6. SAS Institute, Cary, NC).

RESULTS

Visual detection and quantification of conidia in frass. No *Discula*-like conidia were seen in any of the 426 frass pellets collected from the 63 beetles used as controls. The number of *Discula*-like conidia per pellet in frass of beetles exposed to *D. destructiva* ranged from 0 to 3.2×10^6 . The percentage of frass pellets with *Discula*-like conidia fell most rapidly in the first 12 h. Mean number of *Discula*-like conidia per pellet fell gradually for the first 8 h and rose sharply in pellets collected at 10 and 24 h before falling again (Table 1). *Discula*-like conidia were seen in 35% (256/724) of all frass pellets collected. The most conidia discharged by any one beetle 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 (about 75% of these conidia were discharged in the first 12 h). More than 95 and 99% of the conidia counted were discharged by 48 and 72 h, respectively. Four beetles were still discharging frass with *Discula*-like conidia during the 96- to 120-h period. *Discula*-like colonies were isolated from frass of three of these beetles and from frass of a fifth beetle discharged in the 96- to 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 beetles over a 120-h period.

Discula-like conidia were seen in scanning electron micrographs of frass collected in all 2-h intervals, up to 12 h, from beetles exposed to *D. destructiva*. No *Discula*-like conidia were seen in frass of any interval from beetles not exposed to *D. destructiva*.

Viability of conidia in frass. *D. destructiva* was isolated from frass pellets of 76% (31/41) of the beetles 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 beetles carrying viable conidia internally and X = time interval (Fig. 1). The percentage of beetles 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 (Fig. 1). The slopes of the curves were similar, but the initial percentage of beetles carrying conidia was 28.5% higher, and the overall length of time beetles carried conidia was at least 2.5 times longer for beetles on apple than for beetles on aphids (Fig. 1). The percentage of frass pellets from which *D. destructiva* was isolated in each collection interval was consistently higher for beetles fed apple than for beetles fed aphids, with the exception of the 8- to 10-h interval (Table 2). No *Discula*-like colonies were isolated from any of the 454 frass pellets from the 58 control beetles. *D. destructiva* was isolated from 143 of 555 (26%) frass pellets collected from 41 beetles exposed to *D. destructiva*.

Of the beetles fed aphids, *D. destructiva* was isolated from frass of all intervals into the 36- to 48-h interval (Table 2) and from 59% (10/17) of the beetles. *D. 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. Of the pellets from which *D. destructiva* was isolated, 92% and more than 97% had been discharged after 24 and 36 h, respectively.

Of the beetles fed apple, *D. destructiva* was isolated from frass of all intervals (Table 2) and from 87.5% (21/24) of the beetles. *D. 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. Of the pellets from which *D. destructiva* was isolated, 64, 81, 90, and 97% had been discharged after 24, 48, 72, and 96 h, respectively.

Table 1. Visual detection of *Discula destructiva* conidia in frass pellets of 56 convergent lady beetles reared on apple diet following exposure to fungus conidiomata^a

Time after exposure (h)	Frass pellets produced by beetles			
	No. pellets collected	% with conidia	Mean no. conidia per frass pellet ^b	Std. error ($\pm$)
2	23	96	5.63×10^5	1.04×10^5
4	33	82	4.85×10^5	1.08×10^5
6	34	82	4.17×10^5	8.73×10^4
8	28	61	4.01×10^5	1.07×10^5
10	17	71	9.18×10^5	2.51×10^5
12	19	58	6.08×10^5	2.13×10^5
18	40	48	5.05×10^5	1.10×10^5
24	50	42	7.92×10^5	1.42×10^5
36	94	40	4.67×10^5	1.16×10^5
48	65	29	1.70×10^5	5.16×10^4
72	132	17	1.70×10^5	4.80×10^4
96	95	15	4.82×10^4	1.74×10^4
120	94	5	6.00×10^3	1.70×10^3

^a Data based on hemacytometer counts of conidia.

^b Means based on the total number of conidia estimated/number of pellets with *Discula*-like conidia.

DISCUSSION

In previous studies, viable conidia of *D. destructiva* were found internally in convergent lady beetles exposed to the fungus (3–5). The results of this study have shown that large amounts of inoculum can be transported internally by individual beetles and deposited in frass pellets. Ingested conidia of *D. destructiva* can be discharged in viable condition in frass for up to 96 h.

The amount of inoculum beetles ingested and therefore discharged may have been affected by their need for moisture when coming out of storage (10). Having no alternative source of moisture when confined to fungal cultures, beetles may have been attracted to slime droplets of conidia. Beetles were observed ingesting conidia directly from conidiomata and

cleaning conidia-encrusted legs with their mouthparts. However, there was much variation in the numbers of conidia discharged by different beetles within each interval and over the duration of the experiment. Ten beetles had counts of 0 over the entire duration of the quantification experiments, possibly indicating that some beetles either did not ingest conidia, that ingested conidia did not survive passage through the digestive system, or that numbers of conidia in frass were too low to be detected by this method.

The amount of inoculum ingested by individual beetles may have affected the length of time inoculum is carried internally. In a study with *Verticillium albo-atrum* Reinke & Berthold, the time required for the pathogen to be eliminated from the digestive tracts of leaf-chewing

insects was positively associated with the original level of inoculum individual insects ingested (16).

Differences in diet after ingestion of conidia may have contributed to differences in the overall length of time *D. destructiva* was recovered from frass. Other contaminants were more abundant in cultures from frass of beetles on aphids than on apple. Therefore, the isolation of *D. destructiva* from beetles that fed on aphids may be more difficult in later time intervals when the concentration of conidia of *D. destructiva* in frass is lower. However, as beetles generally did not resume feeding until 12 to 24 h after exposure to and ingestion of *D. destructiva* conidia, differences in diet would not explain the differences in the percentages of frass pellets containing viable conidia during the first 6 h (Table 2) and the initial percentages of beetles carrying conidia (Fig. 1).

Apple was used as a food for the beetles instead of aphids for several reasons. Although convergent lady beetles are predaceous, they will eat foods of plant origin when insects are not available (10); in this experiment, they were easily sustained on apple for the duration of the experiment. Frass from beetles on apple contained less insoluble material and could be more thoroughly dissolved for conidial counts. There was less contamination from bacteria and other fungi in frass discharged by beetles on the apple diet, making conidial counts and isolation of *D. destructiva* easier.

Lack of food and water before exposure to *D. destructiva* did not necessarily affect whether beetles would ingest conidia or whether conidia would be discharged in viable condition in frass. Beetles allowed to feed on aphids for 24 and 48 h before exposure to sporulating cultures still ingested conidia of *D. destructiva* and 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.

Although the convergent lady beetle was used in this study as a model insect, *D. destructiva* could be discharged in the frass of other insects as well. Insects that are associated with diseased dogwoods in the field become infested externally with conidia of *D. destructiva* (15), and also may ingest conidia while grooming themselves. Conidia transported internally by insects would not be subjected to external environmental factors that lower viability. Survival of conidia in insect frass offers an additional means by which the distribution of the pathogen can be increased. In greenhouse experiments, convergent lady beetles exposed to sporulating conidiomata of *D. destructiva* and then surface-sterilized were capable of producing symptoms of dogwood anthracnose on healthy dogwood trees (13). Deposits of contaminated insect frass may initiate new infections and en-

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

Table 2. Percentage of convergent lady beetle frass pellets from which *Discula destructiva* was isolated

Time after exposure (h)	Frass pellets produced by beetles on two diets			
	Aphid		Apple	
	No. pellets	% yielding fungus	No. pellets	% yielding fungus
2	5	20 ± 18 ^a	8	100 ± 0
4	14	43 ± 13	16	75 ± 11
6	10	50 ± 16	11	82 ± 12
8	10	50 ± 16	9	56 ± 17
10	8	50 ± 18	5	40 ± 22
12	10	50 ± 16	3	67 ± 27
18	19	21 ± 9	23	65 ± 10
24	17	24 ± 10	22	59 ± 10
36	20	10 ± 7	29	38 ± 9
48	16	6 ± 6	25	28 ± 9
72	50	0	45	20 ± 6
96	48	0	38	21 ± 7
120	53	0	41	7 ± 4

^a Theoretical binomial standard deviation (19).

hance the spread of dogwood anthracnose in natural situations.

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