PATHOGENIC MICROORGANISMS ASSOCIATED WITH THE SOUTHERN PINE CONEWORM (LEPIDOPTERA: PYRALIDAE) ATTACKING LOBLOLLY PINE

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ABSTRACT

Larvae of the Southern pine coneworm, *Dioryctria amatella* (Hulst) (Lepidoptera: Pyralidae), were collected monthly during the growing seasons of 1996 and 1997 from loblolly pine, *Pinus taeda* L., seed orchards in Alabama, Florida, Georgia, South Carolina, and Virginia, and examined for pathogenic microorganisms. One fungus, *Beauveria bassiana* (Bals.) Vuill, a granulosis virus (Baculoviridae: Eubaculovirinae), and a protozoan (phylum Microspora) were found. Five larvae from three localities were infected with *B. bassiana*, 37 larvae from six localities were infected with the granulosis virus, and 69 larvae from 5 locations were infected with the microsporidian. Laboratory trials confirmed that *B. bassiana* and the granulosis virus caused coneworm mortality. *B. bassiana* isolates from all three locations were equally virulent to late instar larvae. Spores of the unidentified microsporidian are free, elongate oval, binucleate and contain 13-14 turns of an isofilar polar filament. The primary sites of infection were the Malpighian tubules and the silk glands. The microsporidian was found in 2 to 51% of larvae sampled. It caused 100% mortality in early instar larvae allowed to feed on artificial diet contaminated with 3×10^3 or 4.5×10^3 spores. More work is needed to determine the importance of these pathogens in regulating populations of southern pine coneworms or their potential utility in an IPM program.

Key Words: loblolly pine, *Pinus taeda*, *Dioryctria amatella*, pathogens, microsporidia, granulosis virus, *Beauveria bassiana*

RESUMEN

Larvas del gusano de las bellotas de pino sureño, *Dioryctria amatella* (Hulst) (Lepidoptera: Pyralidae) fueron recolectadas mensualmente durante las estaciones de crecimiento de 1996 y 1997 en huertos de semillas del pino, *Pinus taeda* L., en Alabama, Florida, Georgia, South Carolina, y Virginia, y fueron examinadas por microorganismos patógenos. Se encontraron un hongo, *Beauveria bassiana* (Bals.) Vuill, un virus granuloso (Baculoviridae: Eubaculovirinae), y un protozoario (Phylum Microspora). Cinco larvas de tres localidades fueron infectadas con *B. bassiana*, 37 larvas de seis localidades fueron infectadas con el virus granuloso, y 69 larvas de 5 localidades fueron infectadas con el microsporidio. Pruebas de laboratorio confirmaron que el *B. bassiana* y el virus granoloso causaron mortalidad en el gusano de las bellotas de pino. Los aislados de *B. bassiana* de las tres localidades fueron igualmente virulentos para las larvas en sus últimos estadios. Las esporas de un microsporidio no identificado son libres, alargadas, binucleadas, y contienen 13-14 vueltas de un filamento isofilar polar. Los sitios principales de infección fueron los túbulos de Malpighi y las glándulas de seda. El microsporidio fué encontrado en un porcentaje del 2 al 51% de las larvas muestradas. Se causó el 100% de la mortalidad en las larvas en los primeros estadios que se permitieron alimentar de una dieta artificial contaminada con 3×10^3 o 4.5×10^3 de esporas. Se necesita hacer más trabajo para determinar la importancia de estos patógenos para regular la poblaciónes de *Dioryctria amatella* o su uso potencial en un programa de Manejo Integrado de Plagas.

INTRODUCTION

Several coneworm species cause severe seed losses in loblolly pine (*Pinus taeda* L.) seed orchards. These include the southern pine coneworm, *Dioryctria amatella* (Hulst); the blister coneworm, *D. clarioralis* Walker; the webbing coneworm, *D. disclusa* Heinrich; and the loblolly pine coneworm, *D. merkeli* Mutuura and Monroe (Yates & Ebel 1975). *Dioryctria amatella* is the most serious of these pests.

Dioryctria amatella feeds on first and second-year cones, rust-infected conelets and terminals of southern pines (Coulson & Franklin 1970, Hedlin et al. 1980). It also attacks fusiform rust galls, *Cronartium quercuum* (Berkely) Miyabe ex Shirai F. sp. *fusiforme,* on stems and branches, and the cambium of tree wounds as alternate hosts necessary for overwintering (Neunzig et al. 1964, Coulson & Franklin 1970, Hedlin et al. 1980). The southern pine coneworm has up to four generations per year in the southern United States (Ebel 1965, Merkel & Fatzinger 1971, Coulson & Franklin 1970, Fatzinger 1981), but most of the damage is caused by progeny of adults that emerge in the spring (Chatelain & Goyer 1980).

Females lay eggs on or near second-year cones (Coulson & Franklin 1970). Once in a cone, larvae develop through five instars (Fatzinger 1970) that feed throughout the cones where they eventually pupate (Neunzig et al. 1964). *Dioryctria clarioralis, D. merkeli* and *D. amatella* often occur in second year cones in the same orchard and several instars of the same species may be present in a single cone (Neunzig et al. 1964, Hanula et al. 1985).

Despite the importance of *D. amatella* and other *Dioryctria* species in limiting seed production, no pathogens have been reported from southern pine coneworms. However, there are records of pathogens associated with other *Dioryctria* species. These include a granulosis virus from *D. abietella* (D. and S.) (Zhimerikin & Gulii 1972) and a nuclear polyhedrosis virus from *D. pseudotsugella* Munroe (Martignoni & Iwai 1986) and *D. sylvestrella* (Kunimi 1993). The fungi *Beauveria bassiana* and *Hirsutella satumaensis* have been recovered from *D. splendidella* H. and L. and *D. sylvestrella,* (Humber & Hansen 2001) and *Metarhizium anisopliae* has been recovered from *D. sylvestrella* in Japan (Kunimi 1993). An unidentified microsporidium was reported from *D. splendidella* in Russia (Sprague 1977).

The objectives of this study were to identify pathogenic microorganisms present in immature stages of southern pine coneworms attacking loblolly pine, their prevalence in natural host populations, and their pathogenicity under laboratory conditions.

MATERIALS AND METHODS

Cones were collected monthly during the summer (July-September) from six seed orchards in 1996 and from seven seed orchards in 1997. In 1996, samples were collected from loblolly pine seed orchards in Escambia and Nassau Counties, Florida; Bibb and Toombs Co., Georgia; Rapides Parish, Louisiana and York Co., South Carolina. In 1997, samples were obtained from orchards in Choctaw Co., Alabama; Escambia Co., Florida; Toombs Co., Georgia; Rapides Parish, Louisiana; York and Dorchester Co., South Carolina; and Albemarle Co., Virginia. Cooperating seed orchard managers collected 50-150 cones per sample from trees scattered throughout their orchards. Samples included almost equal numbers of newly attacked cones (green), older infested cones (brown-green), and old infested cones that were almost dried (brown). The cones were cut open and all larvae and pupae removed. Larvae with external or internal parasitoids were noted along with cadavers. Larvae that appeared healthy were placed individually in small cups containing an artificial diet (Fedde 1982), held at room temperature until they completed development or died, and checked periodically for signs of disease. Those that died were dissected in Ringer's solution (Poinar & Thomas 1978) and examined for disease if no external symptoms were evident.

Larvae that appeared unhealthy, or were damaged while being extracted from cones, were dissected immediately in Ringer's solution. Larval tissues that appeared abnormal during dissection were prepared in wet mount slides and examined with a phase contrast microscope (100-1000×) for pathogens.

Suspected fungal pathogens were isolated either by culturing them from hyphae or spores scraped from the cuticle of cadavers, or by placing whole, surface sterilized cadavers on growth media. Surface sterilization was done by immersing them in a 5% solution of sodium hypochlorite (NaClO) followed by three rinses in sterile water (Poinar & Thomas 1978). After pure cultures were obtained, additional plates were prepared for use in experiments.

We used Sabouraud dextrose agar (SDA) with yeast extract for fungal isolation since it is effective for many entomogenous fungi and the acid reaction (pH 5.6) retards bacterial growth (Poinar & Thomas 1978). We added streptomycin sulfate (0.03g/l) to further inhibit bacterial growth. Cultures were held in a dark growth chamber at 20-25°C for 1-2 weeks for growth and sporulation. Fungi that sporulated in culture were stored for up to 1 month in a refrigerator at 5°C after which new isolates were prepared.

Preparations for microscopic examination were made by growing fungi on cellulose membranes placed on water agar (Alexopoulos &

Beneke 1962). Cellulose membranes (dialysis tubes, Fisher Scientific) were then removed and examined in wet mount preparations after 24 hours, and every 2-3 hours (for 12 hours) thereafter, for diagnostic characteristics (Samson et al. 1988).

Occasionally we were unable to determine a cause of death through dissection and light microscopy. In those cases, the larvae were ground up in Ringer's solution and a drop of the homogenate was placed on a carbon coated grid, stained for 5 minutes with 5% uranyl acetate, and examined by transmission electron microscopy for viral particles. Forty larvae were examined in this way.

Several experiments were conducted to determine the virulence of pathogens recovered from field-infected larvae under laboratory conditions. Coneworms for these experiments were obtain from a laboratory colony of *D. amatella*.

A fungus suspected of causing mortality was tested in one trial to insure that it was the causal agent. Thirty late instar *D. amatella* larvae were inoculated per inoculum density and we tested densities of 0, 6.0×10^4, 1.0×10^5 and 1.6×10^5 spores/µl in sterile water. One 2µl droplet of each spore suspension was placed on the cuticle of the larvae which were then placed in individual cups of artificial diet and held in a growth chamber at 26°C ± 1°C and 92% ± 3% RH until they died or completely developed. Fungi were reisolated from cadavers which exhibited active fungal growth. Pure cultures were then examined and compared to the original cultures to confirm that the same fungus was present and the cause of mortality.

In a second trial, conducted under the same conditions, we compared strains of the same fungus isolated from *Dioryctria* spp. collected from three different localities at a single inoculum density of 4.0×10^4 spores/µl. We also compared mortality following inoculation on the cuticle or *per os*. *Per os* inoculations were done by placing 2µl droplets of 4.0×10^4 spores/µl in a 5% sucrose solution on the mouthparts of CO_2 anesthetized larvae. Cuticular inoculations were the same as before.

To test the granulosis virus, infected, field collected larvae were homogenized in a tissue grinder, diluted with a 5% sucrose solution, and 2l droplets of this inoculum were placed on the mouthparts of anesthetized test larvae. Treated larvae were observed daily and left to develop completely or die. Observations of shape and size of virus particles (virus body) and inclusion bodies through TEM were used to determine the type of virus (Smith 1967).

Microsporidia infections were diagnosed by dissection and light microscopic examination of fresh tissue or stained tissue smears. The latter consist of tissue smeared on slides, air-dried, fixed in methanol (5min), and stained with a 10% Giemsa stain solution (pH 7.4). Larvae anesthetized with CO_2 were inoculated *per os* with 2µl of 1×10^4 microsporidia spores and dissected at 2 day intervals to find which tissues were the primary site of infection. Geimsa stained smears of various tissues were prepared and examined for infection. Since the primary site of infection appeared to be the silk glands, infected portions of that tissue were prepared for electron microscopy. For rapid initial fixation of fresh silk glands, infected tissue was submerged for one hour at 25°C in 2.5% (w/v) glutaraldehyde buffered with 0.1M Na-cacodylate (pH 7.5) to which 5% (w/v) sucrose and 0.5% (w/v) $CaCl_2$ were added. Tissues were post-fixed with 4% (w/v) OsO_4 in cacodylate buffer (pH 7.5) for 1 hour, dehydrated through an ethanol series and then tissues were submerged in propylene oxide twice for 20 min. Tissues were embedded in 812 Epon® plastic (Sabatini et al. 1963). Sections were post-stained with 2% (w/v) aqueous uranyl acetate followed by lead citrate (Harris 1997).

A trial was conducted to determine if the microsporidian caused mortality in *D. amatella* larvae allowed to feed on artificial diet contaminated with spores. Second instar larvae were placed in 1.5 ml microcentrifuge tubes containing a small quantity of artificial diet contaminated with 0, 5×10^2, 2×10^3, 3×10^3 or 4.5×10^3 spores obtained from infected tissues of field collected larvae. Fifty to 60 larvae were treated per dose. The centrifuge tubes were plugged with cotton and the larvae were allowed to feed for 7 d. After 7 d they were transferred to 30 ml capacity diet cups with fresh uncontaminated food. Larvae were monitored every other day until all of the control group pupated. Those that died were dissected and examined for the presence of microsporidian spores. At the end of the experiment all surviving larvae and pupae were examined for microsporidium infections.

Data from laboratory trials were analyzed using the SAS procedure FREQ to analyze contingency tables (SAS Institute Inc. 1987).

RESULTS

We examined 1626 coneworm larvae from six locations during the summers of 1996 and 1997. Of those, 306 (18.8%) were parasitized by other insects, 32 (2.4%) were infected with a granulosis virus, 5 (0.4%) were infected with the fungus *B. bassiana,* and 69 (5.2%) were infected with a microsporidium (Table 1).

The virus was identified as a granulosis type (Baculoviridae: Eubaculovirinae) based the presence of virions occluded individually in granules (Federici 1997). A total of 32 field collected larvae were infected with a granulosis virus at six locations during 1996 and 1997. In 1998, we found 5 larvae infected in a Baldwin Co., Georgia seed orchard. Prevalence ranged from 1 to 14% in field populations (Table 1). Nineteen of 30 late instar

TABLE 1. OCCURRENCE OF PATHOGENIC MICROORGANISMS IN SOUTHERN PINE CONEWORM POPULATIONS.

Sample location Counties and states	year	No. of larvae examined	Occurrence of pathogens: Microsporidia No. (%)	Granulosis virus* No. (%)	*B. bassiana* No. (%)
York, SC	1996	88	0	0	0
Escambia, FL	1996	106	7 (6.6)	1 (0.9)	0
Nassau, FL	1996	89	0	0	0
Bibb, GA	1996	89	0	0	0
Pineville, LA	1996	114	0	0	0
Toombs, GA	1996	410	10 (2.4)	0	0
Toombs, GA	1997	99	12 (12.1)	13 (13.1)	1 (1.0)
Choctaw, AL	1997	74	1 (1.3)	7 (9.5)	1 (1.4)
Escambia, FL	1997	116	17 (14.7)	4 (3.5)	3 (2.6)
York, SC	1997	35	18 (51.4)	5 (14.3)	0
Albemarle, VA	1997	45	0	0	0
Dorchester, SC	1997	34	0	0	0
Rapides, LA	1997	21	4 (19.1)	2 (9.5)	0

Note: Larvae parasitized by insects were not examined for diseases.
*In 1998, 5 additional larvae were found infected with virus in Baldwin CO., GA.

larvae inoculated *per os* with the virus in laboratory trials died, while all of the control group survived to the adult stage. Infected larvae exhibited a variety of symptoms including prolonged development, cessation of feeding, discoloration of the integument (light gray or brown) and a milky white appearance of the hemolymph. The latter was the most reliable diagnostic characteristic for determining infection.

The fungus *B. bassiana* was recovered from *Dioryctria* spp. larvae at three locations, although prevalence was low (Table 1). In laboratory trials, *B. bassiana* (Florida isolate) caused approximately equal mortality at all inoculum densities tested (Table 2). In addition, *per os* inoculations did not increase mortality over cuticular inoculations with the Alabama isolate and we detected no differences in mortality among the three isolates we tested.

Sixty-nine field collected larvae from six locations were infected with a microsporidia (Table 1). Prevalence of this disease organism ranged from 2% in Lyons, Georgia to over 51% at Catawba, South Carolina.

Uninucleate and diplokaryotic vegetative stages were observed in Geimsa stained smears using light microscopy (1000×), but only diplokayotic stages were detected with the electron microscope. Meronts and/or sporonts of the microsporidium were diplokaryotic (Fig. 1A). Spores of the microsporidium observed through light and electron microscopy, were oval, binucleate, contained a single coiled isofilar polar filament with 13-14 turns (Fig. 1B), and measured 5.87 × 2.85 μm (n = 20) in fresh preparations. Spores were not enclosed in any type of sporophorous vesicle and they always occurred individually. Malpighian tubules and silk glands were

TABLE 2. MORTALITY OF LATE INSTAR SOUTHERN PINE CONEWORMS FOLLOWING APPLICATION OF VARIOUS DOSES AND ISOLATES OF *B. BASSIANA* APPLIED TO THE CUTICLE OR *PER OS* TO ESTABLISH THAT THE FUNGUS RECOVERED FROM CADAVERS WAS RESPONSIBLE FOR THE MORTALITY OBSERVED. LARVAE WERE HELD AT 26°C ± 1°C AND 92% ± 3% RH UNTIL DEATH OR COMPLETE DEVELOPMENT.

Isolate/inoculation	dose spores/μl	N	No. dead	Percent mortality
Controls/cuticle	0	30	4	13.3 a
Controls/*per os*	0	30	3	10.0 a
Florida/cuticle	6.00×10^4	30	20	66.7 b
	1.00×10^5	30	23	76.7 b
	1.65×10^5	30	22	73.3 b
Louisiana/cuticle	4.10×10^4	30	21	70.0 b
Alabama/cuticle	4.10×10^4	30	19	63.3 b
Alabama/per os	4.10×10^4	30	20	66.6 b

Note: Percent mortality followed by the same letter are not significantly different (chi-square test, $p < 0.01$).

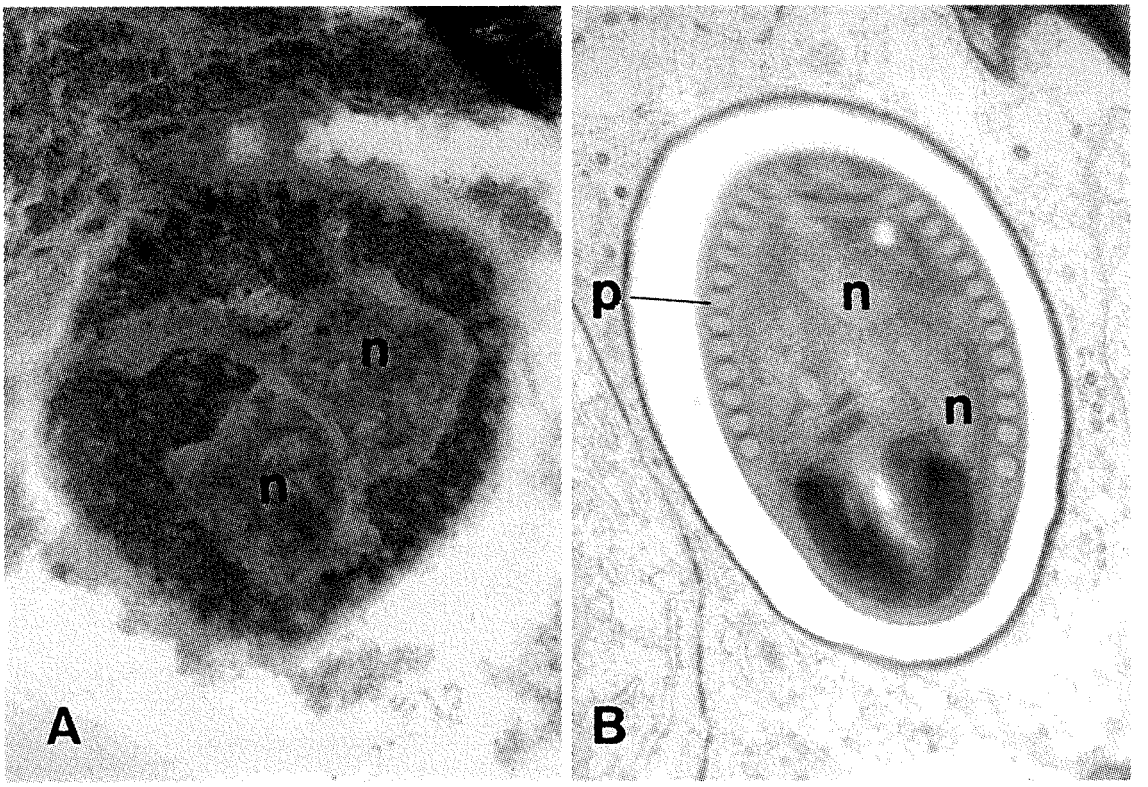

Fig. 1. Electron-micrographs of a microsporidian meront (A) from *Dioryctria amatella* silk gland containing two nuclei (n) in a diplokaryotic arrangement and a binucleate spore (B) showing the polar filament (p) with 13-14 turns. Magnification of A = 40,000× and B = 20,000×.

the primary sites of infection in lightly or newly infected individuals, but we were unable to determine which tissue was infected first. In advanced infections, the microsporidium could be found throughout the host's tissues including the fat body, midgut and epidermis. Larvae reared on artificial diet were successfully infected *per os*. Other means of infection or transmission are unknown.

In laboratory trials, the highest two doses (3×10^3 or 4.5×10^3 spores) caused 100% mortality in larvae exposed in the second instar. Only 45% of the control group survived to pupation but none was infected with the microsporidium.

Discussion

We recovered three pathogenic microorganisms from widely separated populations of the southern pine coneworm. It is likely that these pathogens are found throughout the range of *D. amatella*.

The fungus, *B. bassiana,* is a common pathogen with an extensive insect host list (Tanada & Kaya 1993). Although we only isolated it from five larvae at three locations, laboratory trials showed that it was capable of causing mortality. The low occurrence of *B. bassiana* in field populations may be due to the protected habitat of coneworms. Most cones occur near the tops of pine trees and once *D. amatella* larvae enter cones they rarely leave them, so they are protected from wind-borne pathogens. Vandenberg & Soper (1978) suggested that increased frequency of fungal diseases was due to greater host exposure, favorable physical conditions for fungal spores and the potential for spore accumulation in lower canopy areas. It may be that naturally occurring epizootics of fungal pathogens in coneworm populations are unlikely because larvae occupy protected microhabitats, the majority of loblolly pine cones occur in the tops of trees, and southern pine forests are exposed to frequent periods of hot, dry weather unfavorable to spore longevity outside the soil environment.

This is the first granulosis virus reported from *Dioryctria* spp. in North America, although one was reported from *D. abietella* in Siberia (Zhimerikin & Guli 1972). Baculoviridae are the most commonly observed viral infections in insects with 80% occurring in the Lepidoptera (Evans & Entwistte 1987) and all granulosis virus infec-

tions have been recorded from Lepidoptera (Federici 1997). It is unclear why the granulosis virus is more common than *B. bassiana* in the southern pine coneworm. It may be the virus occlusion bodies or capsules are more persistent in the environment than *B. bassiana* spores. In addition, under laboratory conditions the granulosis virus prolonged larval development while *B. bassiana* killed larvae within a few days. The extended development of viral infected individuals may allow greater viral reproduction (Tanada & Kaya 1993) or it may have increased the likelihood that we encountered infected individuals in our samples.

The microsporidian was the most prevalent pathogen we encountered with up to 51% of the larvae from the York Co., SC population infected with this protozoan. The spores, observed through light and electron microscopy, were oval, diplokaryotic, and had a long, flexible polar filament. Based on characteristics of the spores and the presence of uninucleate and binucleate stages consistent with the description of the type species *N. bombycis* (Sprague et al. 1992) we thought the microsporidian was a member of the genus *Nosema* (Nosematidida: Nosematidae). However, analysis of small subunit ribosomal DNA and comparison to an extensive database of other microsporidia suggests that the microsporidian we found is not closely related to other *Nosema* spp. and is not likely a member of that genus (C. R. Vossbrinck, personal communication). Further genetic analyses, and light and electron microscopy studies are underway to determine the identity and phylogenetic relationships of this species.

Although the microsporidium can kill it's host when they are treated with very high doses, it is unclear what effect this microsporidium has on hosts or host populations under natural conditions. Onstad & Maddox (1989) modeled the effects of *N. pyrausta* (Paillot) on the population dynamics of its pyralid host the European corn borer, *Ostrinia nubilalis* (Hübner). Their results suggest that *N. pyrausta* can regulate *O. nubilalis* populations well below the carrying capacity of its environment. The timing of infection is important in determining what effect a microsporidian has on its host (Onstad & Carruthers 1990). For example, Sajap & Lewis (1992) found that early instars of *O. nubilalis* infected with *N. pyrausta* formed abnormal pupae or adults. Infections in later instars resulted in reduced adult longevity and up to 50% reduction in fecundity. We found that the microsporidium caused mortality at high doses, but we ended our experiment at pupation so we are uncertain if the surviving infected individuals would have developed normally or experienced normal adult longevity. If the nosema-like microsporidian we found has similar effects on its pyralid host, *D. amatella,* then it may be an important factor limiting coneworm populations.

The potential utility of the pathogens found infecting *D. amatella* as biological control agents needs to be determined. The granulosis virus and *B. bassiana* kill their host quickly, and therefore might have potential as biopesticides. *Beauveria bassiana* has the added advantage of being easily cultured. The microsporidian may be important in regulating host populations but additional work is needed to determine its effect. In addition, these pathogens may be useful in biological control programs of other *Dioryctria* spp. Of the three pathogens reported here, only *B. bassiana* was recovered from coneworms in surveys of 5 populations in California and Oregon (Hanula, unpublished data).

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