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Effects of a novel microsporidium on the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae)

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ABSTRACT

A newly discovered microsporidium infecting the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae), provisionally placed in the genus *Canningia*, was studied to determine its impact on *O. sulcatus*. *O. sulcatus* populations from several locations were sampled and evaluated for microsporidiosis. A very low prevalence of the disease was observed in all locations surveyed (<3.0%). Laboratory studies were conducted by orally exposing both larvae and adults of *O. sulcatus* to varying concentrations of *Canningia* sp. spores. Larval bioassays at a variety of dosages (0, 10, etc.) were performed to evaluate pathogen infectivity, larval survival and growth. Adult bioassays (dosages: 0, 10, etc.) were performed to evaluate longevity, fecundity and mechanisms of vertical pathogen transmission. Larvae and adults were infected in all spore treatments. Larval growth was significantly reduced at dosages above 10 spores/larva. Adults infected at all dosages experienced high levels of mortality and fecundity was reduced to zero. Greenhouse trials were performed to determine if larvae feeding in soil acquired infections when spores were topically applied as a drench application (0, 10^5, 10^6, 10^7 spores/pot). Established larvae feeding on plant roots in pots developed infections when exposed to drench treatments of 10^6 and 10^7 spores/pot after 14–21 days. *Canningia* sp. is an acute pathogen of *O. sulcatus* infective to both larvae and adults. Topically applied spores also infected larvae feeding on roots in soilless potting media, suggesting the possibility of using this pathogen in a microbial control program.

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1. Introduction

The black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae) is a univoltine, polyphagous insect that is a severe pest of both field and container-grown ornamentals as well as small fruit crops worldwide (Moorhouse et al., 1992). *O. sulcatus* originated in northern Europe and was first recorded in North America in 1835. The ultimate instar is the primary overwintering stage. In regions where mild winters are typical, a minor proportion of the adult weevil population survives the winter. Pupation and adult eclosion occur in the spring. Adult weevils are nocturnal and cause largely cosmetic damage by notching plant leaves. *O. sulcatus* has a preovipostion period of 20–40 days, feeding on leaves while its reproductive system matures (Smith, 1932). Reproduction is by thelytokous parthenogenesis, so a single individual left unchecked can result in an infestation. Depending on the host plant fed upon by an adult, each individual can lay nearly 900 eggs (Fisher, 2006). Oviposition occurs at night with eggs deposited on the soil surface or inserted into soil crevices (Smith, 1932). Early instars feed on small roots while the later instars feed on larger roots, especially on the phloem and cambium tissues near the soil surface (LaLone and Clarke, 1981). Larval feeding can be quite severe (Moorhouse et al., 1993).

The *O. sulcatus* control program implemented by a majority of small fruit and nursery growers centers on the use of broad spectrum insecticides targeted against preovipositional adults. Infestations are particularly problematic in the ornamental nursery industry in which there is a zero tolerance for *O. sulcatus* infestations. Infested nursery stock cannot be sold and if infested plants are inadvertently shipped, growers risk refusal of the plants by the buyer and will incur the additional return shipping costs and potential loss of future sales. Many of the chemical insecticides currently available for curative applications (i.e. drenches in the late fall and early spring) do not adequately control established *O. sulcatus* larval infestations. Often chemicals applied targeting established *O. sulcatus* larval infestations are not effective against the last instar because they are difficult to apply effectively to large nursery containers (D.J.B., personal observation). Entomopathogenic nematodes are currently available and commercially used as a curative application for *O. sulcatus* control in container-grown nursery stock. Entomopathogenic nematodes are available in a number of different commercial formulations and are most often applied with agricultural sprayers or irrigation equipment (Grewal,

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2002). When applied during periods of favorable soil temperatures, nematodes are efficacious for *O. sulcatus* larval control (Bruck et al., 2005). The nursery industry is continuously looking for new effective alternatives for ridding containers of existing *O. sulcatus* infestations.

Microsporidia are obligately parasitic single-celled eukaryotes that as a group infect insects from nearly every order (Solter and Becnel, 2007). The infective stage of a microsporidium is the mature environmentally resistant spore, which must be orally ingested. Nearly all microsporidia possess a polar filament, a unique infection apparatus that is everted from the spore when infecting the host cell. Most species cause chronic infections, the effects of which can be benign to severe. Commonly seen fitness costs associated with microsporidian infection include reduced longevity and fecundity of adults; increased larval mortality and developmental time; and susceptibility to environmental stressors (Siegel et al., 1986; Bruck et al., 2001; Fuxa et al., 2005; Joudrey and Bjornson, 2007). Microsporidian infection can also have adverse affects on both egg and larval parasiotoids (Cossentine and Lewis, 1987, 1988; Orr et al., 1994; Schuld et al., 1999; Hoch et al., 2000). Relatively few of the currently described microsporidia have been studied extensively for potential use as microbial control agents, primarily due to issues such as complicated life cycles, obligatory parasitism, typically cause chronic rather than acute infections, low persistence in the field and storage difficulties (Brooks, 1988).

A new microsporidium species was recently recovered from a field population of *O. sulcatus* in May, 2003 at a commercial wholesale ornamental nursery located in McMinnville, OR. Initial taxonomic studies show it to be genetically aligned with the *Nosema/Vairimorpha* group (GenBank EU589246), but it lacks one of the major characteristics of the clade, a diplokaryotic nucleus throughout the lifecycle of at least one spore type (L. Solter, D. Bruck, M. Baker, unpublished data). Although genetically basal to the *Nosema*/*Vairimorpha* clade, this microsporidium possesses morphological characteristics (monokaryotic throughout the lifecycle and isolated from beetles) of the relatively recently described genus *Canningia* (Weiser et al., 1995; Kohlmayer et al., 2003), and is provisionally placed in this genus pending further analysis and formal description.

Microsporidia have been isolated from a handful of other closely related *Otiorhynchus* species; all of which were described as belonging to the genus *Nosema* (Hesse, 1905; Weiser, 1951; Sprague, 1977; Hostounsky and Weiser, 1981). These microsporidia were described primarily from infected adults, all of which were collected from the field in Europe and were described prior to the availability of sequence data to assist in their proper placement. Microsporidia previously isolated from otiorhynchids infected a range of host tissues (Hesse, 1905; Weiser, 1951; Sprague, 1977; Hostounsky and Weiser, 1981) and presumably were diplokaryotic (based on the requirement for the genus *Nosema*), while *Canningia* sp. is monokaryotic and infection in *O. sulcatus* larvae is limited to the gut tissues (unpublished data).

2. Materials and methods

2.1. Prevalence in the field

A survey was conducted (2005 and 2006 growing seasons) to determine the natural prevalence of *Canningia* sp. in the field. *O. sulcatus* (larvae and adults) were collected from a number of field sites (small fruit, nursery, and riparian areas) around North America (Table 1). All specimens collected from locations outside of Oregon were stored in the freezer (−20 °C) until shipped. Specimens collected in Oregon were returned to the laboratory and immediately frozen. From each location from which *O. sulcatus* were collected, up to 25 weevils were randomly selected from each collection date and examined for presence of the microsporidium. Because infections are limited to the gut, the entire gut of each weevil was removed to make a fresh tissue smear on a glass slide. Smears were examined at 400× for the presence of *Canningia sp* spores.

Table 1
Prevalence of *Canningia* sp. infection in black vine weevil field collected from various field and nursery locations

Location	Stage evaluated	*n*	Number infected	Percent infection
McMinnville, OR[a]	Larvae/adults	217	6	2.7
Oregon[b]	Larvae/adults	410	1	0.24
New York[b]	Adults	50	0	0
Ont., Canada	Adults	322	5	1.5

[a] Nursery location at which *Canningia* sp. was originally isolated.
[b] Cumulative infection of weevils pooled from various sample locations around the state.

2.2. Larval response and development

Bioassays were performed to determine larval survival and developmental responses to varying dosages of *Canningia* sp. Third instar *O. sulcatus* were obtained from a laboratory colony maintained at the USDA-ARS Horticultural Crops Research Laboratory (HCRL) (Fisher and Bruck, 2004). Spores were produced *in vivo* by infecting third to fourth instar *O. sulcatus* with 10^2 spores and harvested immediately prior (~14 days post-infection) to the initiation of the bioassays. Healthy larvae were individually presented small cubes ($1 \times 1 \times 1$ mm) of meridic diet (Fisher and Bruck, 2004) in 96-well microwell plates. Forty larvae were exposed to diet cubes treated with 0, 10^1, 10^2, 10^3, 10^4 and 10^5 microsporidium spores for 48 h. Twenty larvae that had consumed their entire diet cube were randomly selected and placed individually into 3 oz cups containing fresh meridic diet. Larvae were observed every 2–3 days and mortality recorded. Larvae that died during the course of the experiments were immediately examined for the presence of the microsporidium, as described previously. After 21 days, surviving larvae were weighed and also evaluated for microsporidian infection. An arcsine transformation of the percentage larval mortality was performed to stabilize variance (Snedecor and Cochran, 1989). Data from the bioassays (percent survival and larva weight) were analyzed using the General Linear Models Procedure (GLM) with Tukey's multiple range test used to separate means (SAS Institute, 1999).

2.3. Soil drench applications

Experiments were performed to determine if spores, topically applied to the soil surface of container-grown nursery plants, resulted in larval infection. Spores were produced *in vivo* by infecting third to fourth instar *O. sulcatus* with 10^2 spores and harvested ~14 days post-infection. Rooted cuttings of *Picea abies* 'Nidiformis' were potted into four inch pots with soilless potting media (SB40, Sun-Gro Horticulture, Bellevue, WA) typical of that used in the nursery industry. Pots were artificially infested with five healthy third and fourth instar *O. sulcatus* obtained from the HCRL colony 1 week prior to spore application. At the time that pots were infested, an additional 50 larvae from the same cohort were examined to verify the absence of the microsporidium in the larvae used in the study. No infections were observed. The experiment was arranged in a randomized complete block design with three replications, each containing nine pots, and four levels of spore treatment (0, 1×10^5, 1×10^6 and 1×10^7 spores/pot). Spores were topically applied to each pot in 75 ml of water using a graduated cylinder. Pots