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Variability in growth of *Deladenus siricidicola* on strains of the white rot fungus *Amylostereum areolatum*

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Abstract *Deladenus siricidicola* nematodes are used extensively for biological control of *Sirex noctilio* in the southern hemisphere. They have one morph that is parasitic on *S. noctilio* and another that feeds on the white rot fungus *Amylostereum areolatum* and is used for mass production of the nematode. To examine potential effects of strains of *A. areolatum* found in North America on *D. siricidicola* in a biological control program, first we compared the growth of four isolates of *A. areolatum* on several types of artificial media. We then evaluated the ability of *D. siricidicola* to survive and increase on five isolates of *A. areolatum* and found that nematode populations persisted on all five isolates. One of the slowest growing fungal isolates, ScyME, produced the most nematodes when the fungus was given five and ten days of growth prior to nematode inoculation, while the fastest growing fungus, Aussie, never produced the most nematodes. Although nematodes in all treatments produced eggs, *D. siricidicola* populations were unable to replace themselves when feeding on the fungal isolate SedDF. The differential ability of *D. siricidicola* to persist on different isolates of *A. areolatum* found in North America could affect multiple aspects of a biological control program to control *S. noctilio*.

Keywords Biological control · Woodwasp · Nematode · *Sirex noctilio* · *Sirex* · Invasive species

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Introduction

The nematode *Deladenus* (=*Beddingia*) *siricidicola* Bedding (Tylenchida: Neotylenchidae) has an extraordinary dual lifestyle which alternates between fungal-feeding and parasitic (Bedding 2009). The fungal-feeding form lives in trees and feeds on a white rot fungus, while the parasitic form infects *Sirex* woodwasps, which then carry the nematode to a new tree. Because parasitism often results in sterilization of the female woodwasp, the nematode has been used extensively for biological control of the invasive pest *Sirex noctilio* F. (Hymenoptera: Siricidae) in the southern hemisphere (Hurley et al. 2007).

The woodwasp *S. noctilio* is native to Eurasia and northern Africa and in these regions it is not considered a pest, but it has caused extensive damage since its introduction to New Zealand, Australia, several South American countries and South Africa (Hurley et al. 2007). *S. noctilio* principally infest pine (*Pinus* spp.) trees, into which ovipositing females deposit the symbiotic white rot fungus *Amylostereum areolatum*

(Fr.) Boidin (Russulales: Amylostereaceae) during oviposition. *S. noctilio* larvae develop while feeding on *A. areolatum*-decayed wood and do not survive unless the fungal symbiont is present (Madden and Coutts 1979). An individual from an established population of this woodwasp was first collected in North America, near Lake Ontario, in 2004 (Hoebeke et al. 2005), and in 2005 it was discovered in Ontario, Canada (de Groot et al. 2006). In the United States, *S. noctilio* spread throughout New York and into Pennsylvania by 2006, to Vermont and Michigan by 2007, to Ohio by 2008, and to Connecticut by 2010 (USDA, APHIS 2010).

Female *S. noctilio* that are infected with the parasitic form of *D. siricidicola* are sterilized, but disperse their nematode-filled eggs to new trees, also transporting the fungus needed to sustain the mycophagous form of the nematode (Bedding 2009). The nematodes can have multiple generations in the mycophagous phase. Based on this fact, mass production of *D. siricidicola* in Australia for biological control uses the fungal-feeding form. The *A. areolatum* strain used for mass production has remained in use since its collection from Sopron, Hungary in 1968 (Bedding pers. comm.).

While *Deladenus* species are not particularly insect-host specific, they are fungus specific (Bedding and Akhurst 1978), and although feeding may occur on a given fungus, reproduction may be restricted. Pillai and Taylor (1967), when studying four fungal-feeding nematodes, including *Neotylenchus linfordi*, found that of the ten fungi studied, none of them repelled the nematodes. When they assessed the total nematodes produced on the fungal plates, however, they found that the fungi differed in host suitability, ranging from excellent to poor. Indeed, Hurley et al. (2007) listed fungal-nematode incompatibility as a possible reason for inadequate control of *S. noctilio* in the KwaZulu-Natal region of South Africa. Slippers et al. (2001) speculated that such an incompatibility between nematode and fungal isolate could affect nematode feeding and reproduction when nematodes are introduced to new areas. Given that fungal strain may affect nematode reproduction and that native North American *Sirex* spp. carry different strains of *A. areolatum* (Nielsen et al. 2009), nematode growth on different fungal strains could significantly impact a biological control program involving *D. siricidicola*.

Biological control of *S. noctilio* in the southern hemisphere (Hurley et al. 2007; Bedding 2009; Borchert et al. 2007) has provided a good framework for such an effort in the northeastern United States and Canada. However, several factors complicate direct adoption of the approach used in the southern hemisphere. Unlike the southern hemisphere, where pine trees have been introduced as a managed crop, pines are native to North America (Hurley et al. 2007). Likewise, in the southern hemisphere, the invasive *S. noctilio* is the only siricid, whereas North America hosts several native species of *Sirex* woodwasps that are not considered to be pests (Smith and Schiff 2002; Slippers et al. 2003). Some native North American *Sirex* carry *Amylostereum chailletii* (Fr.) Boidin as a symbiont, although at least one North American *Sirex*, *Sirex* sp. '*nitidus*', is now thought to carry *A. areolatum* naturally at least some of the time (Nielsen et al. 2009). The strains of *A. areolatum* found in native *Sirex* spp. are not the same that are found in *S. noctilio*, based on genetic sequencing data.

With the potential to release *D. siricidicola* in North America, we were interested in whether *D. siricidicola* will feed on the strains of *A. areolatum* present in North America and we hypothesized that strains of *A. areolatum* with faster growth rates would produce more *D. siricidicola*. First, we chose four fungal strains of *A. areolatum*, including the strain used for mass production of *D. siricidicola* in Australia, and determined the relationship between fungal isolate and growth rate using different media. We compared the media used for mass production of *A. areolatum* with types of media used in other studies: Hagem agar (Stenlid 1985), potato dextrose agar amended with thiamine, and green bean agar (GBA, Thomsen and Koch 1999). Next, we grew five strains of *A. areolatum* on one type of media and then inoculated the fungal cultures with *D. siricidicola* to investigate the relative increase of the nematodes on the different strains of fungus.

Materials and methods

Fungal isolates

The geographic and host origins of the five strains of *A. areolatum* included in this study are listed in Table 1. Four of the strains were isolated from *Sirex* collected in North America and these were chosen to represent the diversity of *Sirex* hosts and of genotypes

Table 1 Isolates of *A. areolatum*

Isolate ID	Isolation from	Original host	Date Isolated	IGS strain[a]
Aussie	Sopron, Hungary	*S. juvencus*	1967–1968	BDF
OtisAa	Oswego County, NY, USA	*S. noctilio*	Jan 2006	D
ScyME	Waldo County, ME, USA	*S. nitidus*	13 Sep 2007	BE
Gr94-1	Fulton County, NY, USA	*S. noctilio*	19 Feb 2008	BD
SedDF	Oswego County, NY, USA	*S. edwardsii*	19 Sep 2007	D

[a] IGS region strains as reported in Nielsen et al. (2009)

present in North America, based on intergenic spacer regions (IGS) (Nielsen et al. 2009). One of these isolates is thought to be native (ScyME) and three are thought to be introduced (OtisAa, Gr94-1, and SedDF). The fifth fungal strain (Aussie) was isolated from *Sirex juvencus* (L.), collected in Hungary and maintained in culture since 1967–1968. This strain was obtained from Ecogrow Environment (Queanbeyan, NSW, Australia) where it is used for mass production of the *S. noctilio* biological control agent *D. siricidicola*.

Nematode isolate

The strain of *D. siricidicola* used for these studies was obtained from Ecogrow Environment, where it is mass produced for biological control. The nematodes were obtained from Ecogrow in 2006. This strain originated, along with the Aussie strain of *A. areolatum*, in Sopron Hungary. However, due to the loss of nematode infectivity following years of laboratory culture, the strain of nematode was reisolated from an early field site where it was released in Tasmania (Bedding 2009). Nematodes were kept under USDA, APHIS permit in a quarantine facility (the Sarkaria Arthropod Research Laboratory, Ithaca, New York, USA) for the duration of the experiment. Nematodes were initially grown on *A. areolatum* Aussie using half-strength potato dextrose agar (Difco, Sparks, MD, USA) with a total of 25 g l^{-1} agar to make this medium harder (½PDAh) (Bedding pers. comm.). Petri dishes with nematodes were maintained inside brown paper bags in a 23 °C incubator with no light.

Fungal growth assay

The four isolates of *A. areolatum* were cultured on four types of media: GBA (Dhingra and Sinclair 1995), Hagem agar (Hagem) (Stenlid 1985), potato dextrose agar plus 5 mg l^{-1} thiamine hydrochloride (PDAth) (Thomsen and Koch 1999), and ½PDAh. This latter medium is regularly used for maintaining this fungus commercially and therefore was considered a control. During experiments, cultures were maintained at 23 °C without lights.

For each fungal strain, a plug was taken at the growing edge of a fungal colony, normally grown on ½PDAh, using a sterile 3 mm diameter cork borer and transferred to the center of a 100 mm diameter petri dish of the test media. In a few instances, to encourage the growth of enough fungus for the experiment, plugs were taken from PDAth. Fungal plugs were allowed four days to begin growing on the new media before measurements began. Culture diameter was measured along two perpendicular axes every 48 h until either the fungal growth had filled the plate or had stopped growing for two measurement intervals (up to 44 days of observations). For each measurement interval, the area occupied by the fungal colony was calculated from the average of the two diameter measurements. This study was replicated 4–5 times with one dish per treatment for each replicate.

Nematode propagation assay

All nematode growth assays used the ½PDAh medium for growing *A. areolatum* and were conducted at 23 °C in darkness. The five strains of *A. areolatum* were grown in 60 mm diameter Petri dishes by transferring a 3 mm diameter plug from the edge of a culture. The fungus was then allowed to propagate for one, five, or ten days at 23 °C in darkness, at which point each dish was inoculated with nematode eggs. To inoculate plates with eggs, *D. siricidicola* colonies were flooded with distilled water and the liquid containing nematodes and eggs was filtered three times through a Swinnex filter

holder (Millipore) equipped with a 60 μm filter, which allowed eggs to pass through into the filtrate. In some instances, it was unavoidable that a few small juveniles passed through with the eggs, but this happened equally for all treatments. Ten 20 μl samples were taken from this stock suspension and all eggs were counted under a dissecting microscope at 20× magnification to volumetrically determine the total number of eggs in the stock solution. The solution was then diluted so that a mean of 167 eggs were present per 20 μl drop. Three drops were added to each fungal dish, equidistant from one another and half-way between the fungal plug and the edge of the Petri dish. Therefore, each dish received approximately 500 eggs. After 25 days, the dishes were flooded once with water and ten 20 μl samples of each of the washings were examined under a dissecting microscope to volumetrically determine the total number of eggs and living nematodes per dish. The amount of water used for flooding differed by plate due to the varying amount of fungus and nematode density, but it was always measured for use in calculation of the total nematodes produced per plate. This study was conducted on two separate dates, with three replicates of each treatment on the first date and eight replicates of each treatment on the second date.

Data analysis

To calculate daily fungal growth, only data points from the log growth phase of fungal cultures were used. As a lower limit, we used the interval when the culture was >8 mm in radius. Because cultures did not always grow to the Petri dish edges, we considered that fungal growth had stopped when the colony had grown 0.5 mm or less for two consecutive measurement intervals. Fungal growth rate (mm day^{-1}) was calculated by dividing the final diameter by the number of days of growth (during the aforementioned period). For analysis, fungal growth rates were transformed to log (x + 1) to evaluate the effects of media and isolate using a two-way general linear model (SAS 2002–2008). To compare individual isolates or medium, least mean square tests were conducted using the Bonferroni correction to maintain family-wise error rates at an alpha = 0.05.

To compare the increase in nematode numbers when feeding on different fungal isolates, the numbers of nematodes (including both eggs and living nematodes) produced after 25 days were transformed to log (x + 1). Fungal isolate and time of fungal growth before nematode inoculation were main effects using a multifactorial ANOVA and means were separated with Tukey's HSD (JMP 1989–2007). In a separate analysis, only the numbers of eggs produced after 25 days were log-transformed and analysed using the same model as for total nematodes.

Results

Growth of fungal isolates

The interaction between media and fungal isolate was significant ($F_{12,53} = 2.82$; $P = 0.0046$) so separate analyses were conducted comparing growth of each isolate on different media. When comparing isolate growth on each type of media, the fungal isolate used for mass production of *D. siricidicola*, Aussie, grew faster than other isolates on each of the media tested (Table 2). Rates of growth for the other three isolates did not differ significantly on GBA or Hagem agar and growth rates for isolates other than Aussie differed inconsistently on ½PDAh and PDAth (Table 2).

Each isolate varied in growth rate on different media. For each isolate, growth was consistently fast on GBA and slow on Hagem agar (Table 2). On Hagem agar, the commercial isolate Aussie grew to the edges of Petri dishes but other isolates stopped growing at 26.4–29.6 mm radius, which in all cases was significantly less growth than Aussie (Aussie vs. Gr94-1: $t = 7.4564$, df = 1, $P < 0.0001$; Aussie vs. ScyME: $t = 5.8563$, df = 1, $P < 0.0001$; Aussie vs. SedDF: $t = 7.7891$, df = 1, $P < 0.0001$). During this study, we compared two adaptations of potato dextrose agar: ½PDAh *versus* PDAth. Growth rates on these media did not differ significantly for any isolate although for three of the four isolates, growth was numerically greater on PDAth than on ½PDAh. The fungal isolate from North American *S. nitidus* mycangia, ScyME, grew more slowly on all media compared with GBA (Table 2).

Nematode growth on fungal isolates

All *A. areolatum* isolates supported some growth of *D. siricidicola*. There was a significant interaction between the isolate and the time of fungal growth before nematode inoculation ($F_{8,152} = 2.31$; $P = 0.02$), so

Table 2 Mean (±SE) millimeters per day fungal growth of four *A. areolatum* isolates growing on four different media

Media[b]	*A. areolatum* isolates[a]			
	Aussie	Gr94-1	ScyME	SedDF
Green bean agar (GBA)	4.7 ± 0.4 ab A	2.5 ± 0.2 a B	2.7 ± 0.3 a B	1.9 ± 0.1 a B
Hagem agar	3.3 ± 0.4 b A	0.9 ± 0.1 c B	1.0 ± 0.1 b B	0.9 ± 0.1 b B
½ Strength potato dextrose agar, increased to 2.5 % agar (1/2PDAh)	4.2 ± 0.1 ab A	1.1 ± 0.1 bc C	1.0 ± 0.1 b C	1.8 ± 0.1 a B
Potato dextrose agar + thiamine (PDAth)	4.9 ± 0.3 a A	1.9 ± 0.5 ab B	1.1 ± 0.1 b C	1.7 ± 0.1 a BC

[a] Different lower case letters by columns denote differences between mean fungal growth rates among types of media within isolate, and upper case letters denote differences across rows among isolates within media type (least square means using the Bonferroni correction to split the $\alpha = 0.05$ across tests)

[b] Media described in the "Materials and methods" section

subsequent analyses were performed to see which isolates in particular were significantly affected by days of fungal growth. Isolates for which days of growth was a significant factor were Aussie ($F_{2,31} = 3.45$; $P = 0.04$), OtisAa ($F_{2,30} = 4.06$; $P = 0.03$), and ScyME ($F_{2,26} = 12.99$; $P = 0.0001$). In general, nematodes grown on OtisAa and ScyME produced more offspring as the number of days of fungal growth prior to inoculation increased (Fig. 1). The slowest growing isolate, ScyME, when given ten days to grow prior to nematode inoculation, produced more nematodes ($4.7 \times 10^4 \pm 1.0 \times 10^4$; mean ± SE) than any other treatment given ten days of fungal growth prior to inoculation with nematodes (Fig. 2c). Isolates Gr94-1 ($F_{2,28} = 1.37$; $P = 0.27$) and SedDF ($F_{2,31} = 1.35$; $P = 0.28$) were not affected by days of fungal growth prior to inoculation. Isolate SedDF did not produce more nematodes than the initial inoculation in any treatment, despite nematodes developing to adults and reproducing (Fig. 1).

The total number of eggs produced had a significant interaction between fungal isolate × days of growth ($F_{8,152} = 3.07$; $P = 0.003$), so separate analyses were conducted. Nematodes produced eggs in all treatments (Fig. 3), although there was high variability between treatments. As with the total nematodes produced, ScyME produced numerically the most eggs with 10 days of fungal growth prior to inoculation compared with all other treatments ($3.3 \times 10^4 \pm 7.6 \times 10^3$). For three isolates (Aussie, OtisAa, and SedDF), the number of days of fungal growth prior to inoculation did not affect the number of eggs present at 25 days (Fig. 3). Curiously, the number of eggs for Gr94-1 declined by the ten days treatment while for ScyME the number of eggs increased in the ten days treatment. Numerically, the fewest eggs (27 ± 8) were produced when SedDF was grown for ten days prior to inoculation with nematodes.

When all fungal isolates were given one day to grow prior to nematode inoculation, numbers of nematodes produced varied, although the fewest nematodes were produced on SedDF cultures (Fig. 2). Once the fungal isolates were given five or ten days to grow, however, the numbers of nematodes in plates with Aussie, Gr94-1, and OtisAa were not significantly different from each other. In contrast, ScyME produced more nematodes when the fungus was given ten days rather than five days to grow prior to inoculation with nematodes ($F_{2,16} = 24.7$; $P = 0.0001$). Nematodes feeding on SedDF grew poorly in all treatments. However, it should be noted that although the number of nematodes grown on SedDF after 25 days did not exceed the initial inoculation of 500, nematodes feeding on this isolate laid eggs and the eggs hatched, so some degree of reproduction was possible. Experimental replicate had no significant effect ($F_{1,152} = 0.39$; $P = 0.5351$).

Discussion

Deladenus siricidicola was able to persist when feeding on all *A. areolatum* isolates included in this study, despite the fact that growth rates for fungal isolates differed. However, nematode population numbers on the different fungal isolates varied enormously, ranging from very few nematodes produced (SedDF) to a nearly 100-fold increase in population

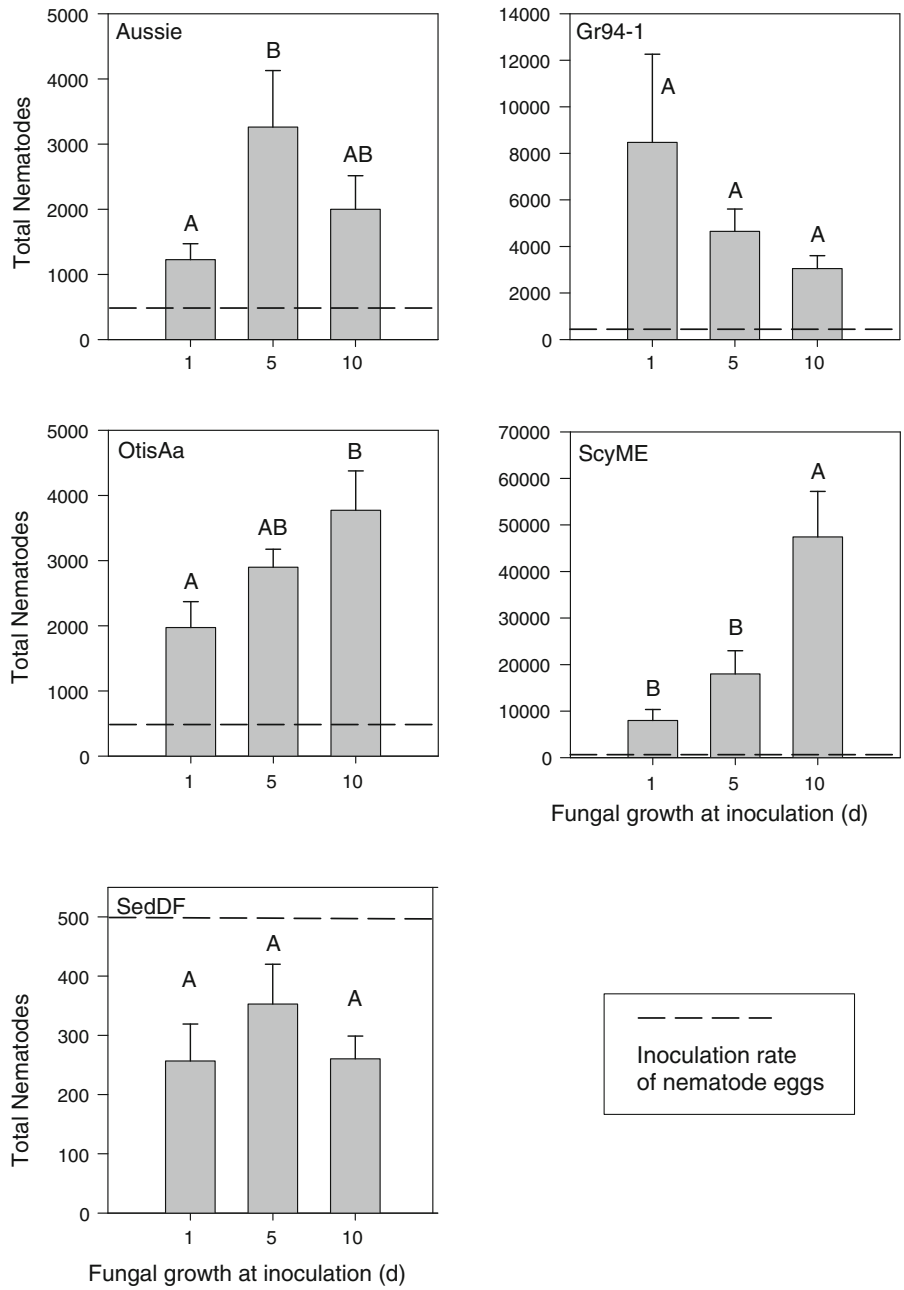
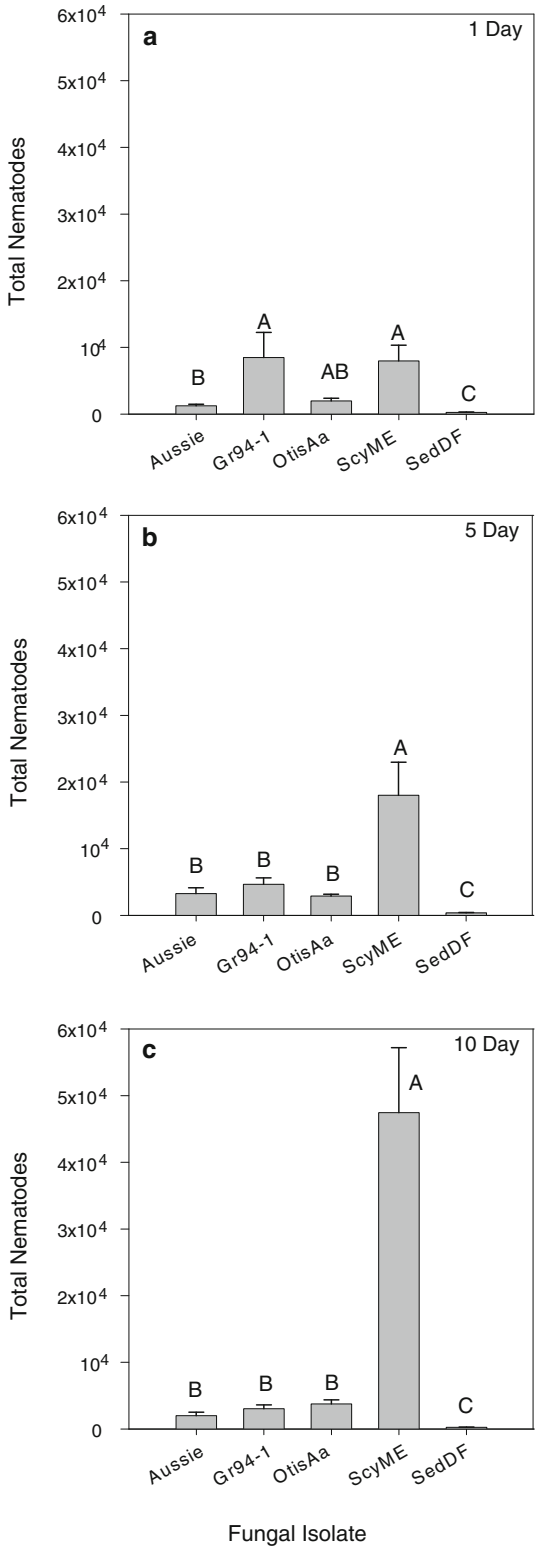


Fig. 1 Mean nematodes (+SE) produced per Petri dish after 25 days for five isolates of *A. areolatum* inoculated with nematodes after one, five, and ten days of fungal growth, by isolate. Dashed line indicates the initial number of nematodes inoculated per dish. Different upper case letters denote differences between mean numbers of nematodes produced (separations given by Tukey's HSD test). Note differences in *y* axes

(ScyME). Some fungal strains established in North America supported more nematode propagation than the commercial fungal strain. Interestingly, the fungal isolate that produced the most nematodes (ScyME) is considered to be native to North America.

Deladenus siricidicola only feeds on *A. areolatum* (Bedding and Akhurst 1978; A.E.H. and S.J.L. unpublished data) but *A. areolatum* occurs as different strains. The strains that *D. siricidicola* could potentially encounter in North America would depend on



◀ **Fig. 2** Mean nematodes (+SE) produced after 25 days for five isolates of *A. areolatum* inoculated with nematodes after one, five, and ten days of fungal growth, by day. **a** one Day, **b** five days and **c** ten days. Different upper case letters denote differences between mean numbers of nematodes produced (separations given by Tukey's HSD test)

those strains being carried by the resident *Sirex* species. *S. noctilio* alone carries at least two strains of *A. areolatum* in North America (Nielsen et al. 2009; Bergeron et al. 2011). North American *Sirex* species were previously thought to carry only *A. chailletii* as their symbiotic fungus (Gilbertson 1984; Smith and Schiff 2002). However, the North American native species *Sirex* sp. '*nitidus*' has been collected carrying a strain of *A. areolatum* that is presumed to be native to North America (IGS BE strain) (Nielsen et al. 2009). When evaluating the potential for a biological control program using *D. siricidicola* in North America, the ability of the nematode to persist when feeding on native North American *A. areolatum* must be understood. If *D. siricidicola* is introduced into a tree where the native *A. areolatum* is established, survival and increase of the nematode would depend on its use of the native *A. areolatum* strain. Our study demonstrates that the commercial *D. siricidicola* grows well when feeding on the native strain of *A. areolatum* (ScyME), suggesting that presence of this fungal strain in trees would potentially promote growth of this biological control agent. In addition, *D. siricidicola* grew well on one IGS strain of *A. areolatum* isolated from *S. noctilio* in North America (BD), although growth on the D strain of IGS (SedDF vs. OtisAa) carried by *S. noctilio* in North America was variable.

Cohabitation of the invasive and native *Sirex* species within trees, along with the ability of *D. siricidicola* to increase when feeding on different isolates of *A. areolatum*, could lead to close proximity of this biological control agent with native species of *Sirex*. Whether the commercial strain of *D. siricidicola* will parasitize the native *Sirex* is not known. Although the commercial strain of *D. siricidicola* has not been introduced into North America, a strain of *D. siricidicola* has been found in North America which parasitizes *S. noctilio* without causing female sterilization (Yu et al. 2009). An asynchrony between host and parasite physiology results in nematodes being present in the woodwasp ovaries but not within the

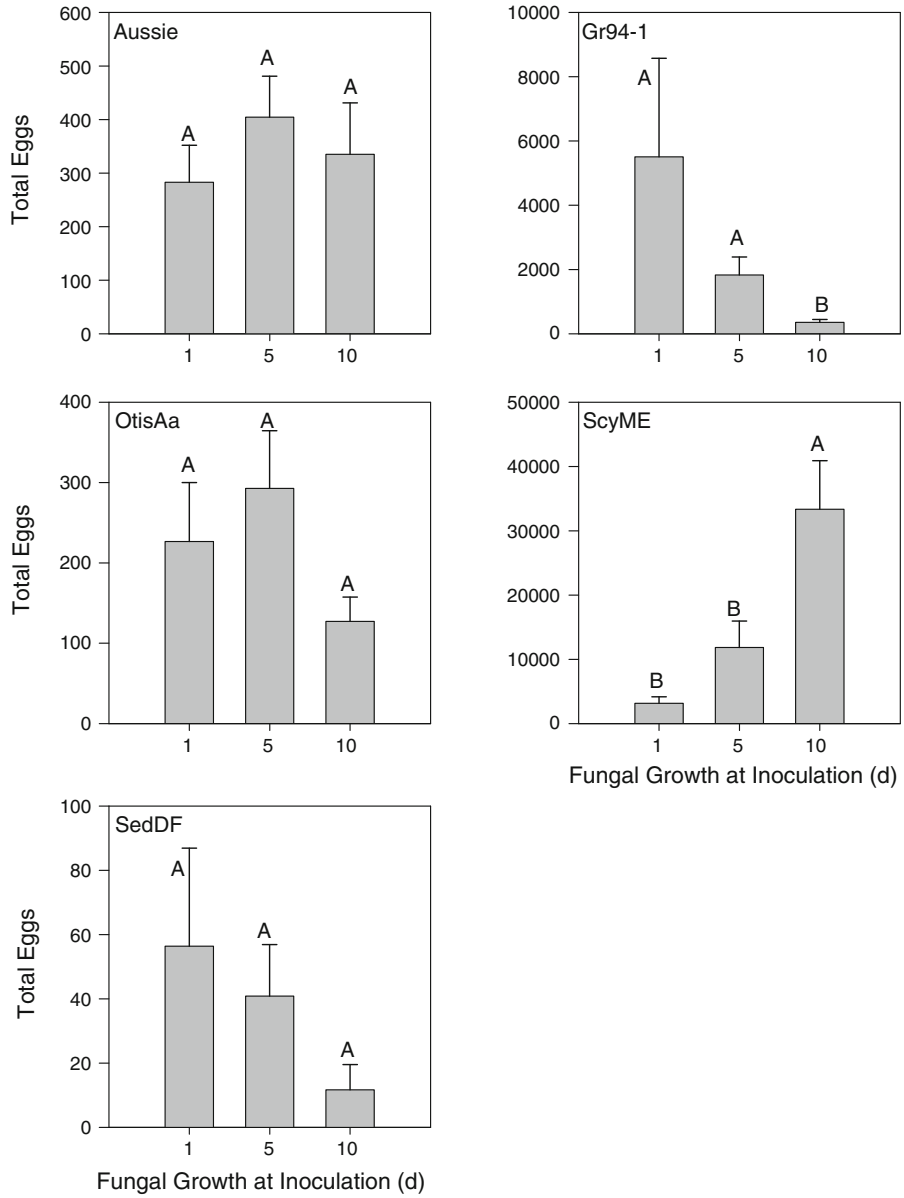


Fig. 3 Mean nematode eggs (+SE) produced after 25 days for five isolates of *A. areolatum* inoculated with nematodes after one, five, and ten days of fungal growth, by isolate. Different upper case letters denote differences between mean numbers of nematodes produced (separations given by LSMeans Tukey HSD test). Note differences in *y* axes

woodwasp eggs (Zondag 1975; Yu et al. 2009; D.W.W. unpubl. data). Thus, even if *D. siricidicola* parasitizes native North American *Sirex* woodwasps, it is not known if such an asynchrony between host and parasite physiology would occur in these hosts.

Contrary to our initial hypotheses, the two slowest growing fungal isolates produced the most nematodes. Bedding (1972) stated that because the mycophagous form of the nematodes only feed on the growing hyphal tips of a fungal colony, to grow *D. siricidicola* a balance must be achieved so that the *A. areolatum* culture does not overgrow the nematodes. In exploring the sides of the nematode/fungus "balance," our study failed to find a scenario in which the nematodes ate all of the fungal tips and then starved. However, nematode production on Aussie, the fastest growing isolate,

may be evidence of the other side of the balance, where the fungus out-grew the nematodes, reaching the edge of the plate because nematode populations did not increase fast enough to stop or slow the rapid fungal growth. A limitation of our study is that it took place in Petri dishes and therefore could not take into account interactions that may occur within fungal colonies in flasks used for commercial production of nematodes or when nematodes and fungus are growing within trees. Although *D. siricidicola* may be inoculated into a tree with the Aussie isolate of *A. areolatum*, Aussie would not necessarily become the prevalent fungus established in the tree. While Aussie has been inoculated into pine trees in the southern hemisphere along with *D. siricidicola*, it has not been recovered thus far in isolations from either the wood or from emerging *S. noctilio*, although a systematic search was not made for it (Slippers pers. comm.). Additionally, it is not known how *D. siricidicola* and *Sirex* spp. will interact in trees hosting multiple isolates of *A. areolatum* or whether the nematodes would preferentially respond to different fungal isolates.

Vasiliauskas et al. (1998) used Hagem agar for growing *Amylostereum* species but for our studies, this medium consistently produced slow fungal growth. Our studies demonstrated that often fungal growth on GBA was faster than on other media, although differences were not always significant (Table 2). In studies in Denmark, *A. areolatum* was first grown on GBA to speed up growth before being moved to PDA, which produced slower growth (Thomsen and Koch 1999). While we did not test full strength PDA for comparison, the addition of thiamine to full strength PDA did not significantly increase growth when compared with ½PDAh. Thomsen and Koch (1999) reported that the addition of thiamine to PDA resulted in growth equivalent to GBA, but in our study this was only true for three of four isolates. The fungal isolate ScyME grew faster on GBA than on PDA plus thiamine.

The ability of *D. siricidicola* to differentially survive and propagate on different strains of *A. areolatum* could affect numerous aspects of a biological control program for *S. noctilio* using *D. siricidicola*. First, fungal isolates could be chosen to optimize mass production of *D. siricidicola*, based on the ability of the nematodes to increase in population numbers on a given isolate. Second, if *D. siricidicola* in a tree encounters alternate isolates of *A. areolatum* that have already colonized parts of that tree, the ability of the nematodes to persist on the established strain of *A. areolatum* could lead to success or failure in parasitizing *S. noctilio* within that tree. Lastly, the same ability of *D. siricidicola* to grow and increase when feeding on a given fungal isolate of *A. areolatum* could lead to non-target effects if *D. siricidicola* has the ability to parasitize and is in proximity with the native siricids associated with *A. areolatum* in the same tree.

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