BioControl (2018) 63:739–749
https://doi.org/10.1007/s10526-018-9897-1

The fungal matrices of *Ophiostoma ips* hinder movement of the biocontrol nematode agent, *Deladenus siricidicola*, disrupting management of the woodwasp, *Sirex noctilio*

F. Yousuf · A. J. Carnegie · R. Bashford · H. I. Nicol · G. M. Gurr

Received: 12 October 2017 / Accepted: 25 June 2018 / Published online: 3 July 2018

Abstract *Deladenus* (= *Beddingia*) *siricidicola* (Tylenchida: Neotylenchidae) is the most effective biocontrol agent used against the invasive wood wasp, *Sirex noctilio* (Fabricius) (Hymenoptera: Siricidae). The nematodes feed and reproduce on the wood-inhabiting fungus, *Amylostereum areolatum* (Chaillet ex Fr.) Boidin (Russulales: Amylostereaceae) and parasitise larvae of *S. noctilio.* In the nematode biocontrol program, the nematodes are inoculated into herbicide-weakened 'trap trees'. Recent declines in nematode parasitism of *S. noctilio* in Australia have coincided with an increased incidence of an exotic bark beetle, *Ips grandicollis* (Eichhoff) (Coleoptera: Curculionidae), attacking trap trees and vectoring a wood-inhabiting fungus, *Ophiostoma ips* (Rumbold) Nannfelt (Ophiostomatales: Ophiostomataceae), which may inhibit migration of the nematode within the tree to the detriment of *S. noctilio* biocontrol. Several in vitro and in vivo experiments were conducted to investigate the effect of fungal interactions on the ability of *D. siricidicola* to locate and reproduce on *A. areolatum. Deladenus siricidicola*

Handling Editor: Ralf Ehlers

F. Yousuf · G. M. Gurr (✉)
Primary Industries, Graham Centre for Agricultural Innovation, Charles Sturt University, P.O. Box 883, Orange, NSW 2800, Australia
e-mail: fazila_yousuf@hotmail.com

G. M. Gurr
e-mail: ggurr@csu.edu.au

A. J. Carnegie
NSW Department of Primary Industries, Forestry, Level 12, 10 Valentine Avenue, Locked Bag 5123, Parramatta, NSW 2151, Australia
e-mail: angus.carnegie@dpi.nsw.gov.au

R. Bashford
Forestry Tasmania, 79 Melville Street, Hobart, TAS 7000, Australia
e-mail: dick.bashford3@gmail.com

H. I. Nicol
Dalyup Statistical Consulting, P.O. Box 8773, Orange, NSW, Australia
e-mail: helen.i.nicol@gmail.com

G. M. Gurr
Institute of Applied Ecology, Fujian Agriculture and Forestry University, Fuzhou, China

showed preference to *A. areolatum* in the presence and absence of *O. ips,* but the presence of *O. ips* negatively affected the choice response and the number of eggs laid by the nematodes. *Deladenus siricidicola* was unable to survive and reproduce on *O. ips.* Results give a clearer understanding of the choice response of *D. siricidicola* in *I. grandicollis* infested trees, explaining the disruptive impact of bark beetles on biocontrol of *S. noctilio,* an effect that could extend from Australia to other important pine growing countries.

Keywords Chemotaxis · *Beddingia siricidicola* · Entomopathogenic · Ophiostomataceae · Trap trees · Neotylenchidae

Introduction

The parasitic nematode, *Deladenus* (= *Beddingia*) *siricidicola* (Bedding) (Tylenchida: Neotylenchidae), is the major biocontrol agent of a serious woodwasp pest, *Sirex noctilio* (Fabricius) (Hymenoptera: Siricidae) and is one of the most successful classical biocontrol projects of its kind (Bedding and Iede 2005). Other biocontrol agents available include parasitic wasps, *Ibalia leucospoides* ensiger (Norton) (Hymenoptera: Ibaliidae), *Megarhyssa nortoni* (Cresson) (Hymenoptera: Ichneumonidae), two species of *Rhyssa* (Hymenoptera: Ichneumonidae), and *Schlettererius cinctipes* (Cresson) (Hymenoptera: Stephanidae) (Taylor 1976). In the nematode biocontrol program, typically 'trap tree plots' consisting of ten trees are annually set up in the most susceptible age-class areas in a forest where the *S. noctilio* population is likely to be prevalent (Bedding and Iede 2005; Carnegie and Bashford 2012). Nematodes later are inoculated into the *S. noctilio*-infested trap trees. The nematodes are extraordinary as they have two independent life cycles with two different life forms: free-living and parasitic (Bedding 1972). In its free-living (mycetophagous) form the nematode feeds and breeds on *Amylostereum areolatum* (Chaillet ex Fr.) Boidin (Russulales: Amylostereaceae), a symbiotic fungus of *S. noctilio* deposited in tree sapwood at the time of oviposition (Bedding 1967, 1972). This free-living form molts into preparasitic adults that mate, then gravid females penetrate *S. noctilio* larvae, beginning the parasitic cycle (Bedding 1967). The female parasitic nematodes release juveniles within the pupa of the host which invade the host ovaries and testis rendering *S. noctilio* females sterile (Bedding 1967, 1972). Parasitized *S. noctilio* female adults emerge and disperse across the forest laying sterile 'eggs' containing juvenile nematodes (Bedding and Iede 2005; Bedding 1972).

In Australia, this method of biocontrol of *S. noctilio* has been successful, resulting in almost 100% parasitism in some regions (Bedding 2009). However, in recent years, a decline in the effectiveness of this program has been observed, coinciding with an increase in the numbers of the exotic bark beetle, *Ips grandicollis* (Eichhoff) (Coleoptera: Curculionidae) (Carnegie and Bashford 2012; Gitau et al. 2013). *Ips grandicollis* attacks trap trees and vectors a sap-staining fungus, *Ophiostoma ips* (Rumbold) Nannfelt (Ophiostomatales: Ophiostomataceae). *Ophiostoma ips* is a fast-growing fungus and competes with *A. areolatum* in the wood (Yousuf et al. 2014a, b). Previous studies have shown that trap trees infested by *I. grandicollis* had a greater volume of *O. ips* than *A. areolatum* and produced more unparasitised *S. noctilio* than trees with no *I. grandicollis* infestation (Yousuf et al. 2014a). The survival and reproduction of the free-living nematodes depends on the presence of *A. areolatum*. For biocontrol to be successful the larvae of *S. noctilio* must become infected with the nematodes. Consequently, it is crucial for the nematodes to locate *A. areolatum* when released in the trap trees. It is not yet known if *D. siricidicola* has the ability to distinguish, locate and aggregate on *A. areolatum* and insect hosts in the presence of *O. ips*.

Nematode behaviours (e.g., attraction, repellence, movement, penetration, feeding, mating, inhibition and hatching stimulation) (Bilgrami and Gaugler 2004a, b; Boender et al. 2011) arise as a response to various external stimuli such as physical or chemical gradients and environmental structure (e.g., CO_2, vibration, temperature, chemical compounds, electromagnetic stimuli) (Shapiro-Ilan et al. 2014). Nematodes have a variety of different sensory organs with which they can perceive cues (e.g., chemical, electrical, light, mechanical, and temperature) from their environment (Zuckerman and Jansson 1984; Grewal et al. 1993; Jones 2002) to orient, move, and locate a sexual partner, energy source (food), and host (Lee 2002). Chemotaxis is the directed orientation of the nematode toward or away from the source of

stimulation. It is the primary means by which nematodes locate their host (Zuckerman and Jansson 1984; Grewal et al. 1993). Nematodes can detect chemical compounds released from the hosts on which they feed and reproduce (Klink et al. 1970; Gaugler et al. 1980; Grewal 2000; Rolfe et al. 2000; Murayama and Maruyama 2013). Stimuli can cause attraction or aggregation behaviour, for example, the fungal feeder, *Neotylenchus linfordi* (Hechler) (Neotylenchidae: Nematoda), is attracted to and aggregates at fungal mycelia secretions (Klink et al. 1970). Repulsion or avoidance is another type of behaviour, for example, the juvenile nematodes of *Meloidogyne incognita* (Kofoid and White) Chitwood (Tylenchida: Heteroderidae) are repelled from nematophagous fungi, *Monacrosporium cionopagum* (Drechsler), *M. ellipsosporum* (Preuss) (Helotiales: Orbiliaceae) and *Hirsutella rhossiliensis* (Pat) (Hypocreales: Ophiocordycipitaceae) (Robinson and Jaffee 1996).

In light of the above, we sought the answers to the following questions: (1) Can *D. siricidicola* distinguish, locate, aggregate, and establish on *A. areolatum* in a dual culture of *O. ips* and *A. areolatum*; (2) does *D. siricidicola* feed and lay eggs on *O. ips*; and (3) can *D. siricidicola* move through *O. ips*-infected wood?

Materials and methods

Nematode and fungal cultures

The nematode culture of *D. siricidicola* was purchased from Ecogrow Environment Pty Ltd (Australia) in 2013–2014. These nematodes were of the Kamona strain mass reared on *A. areolatum* in the laboratory. Inoculating mixture of the nematodes was prepared by suspending one million nematodes in 1% polyacrylamide gel. The agar cultures of *D. siricidicola*, *O. ips* (DAR 82118) and *A. areolatum* (DAR 82117) were used from previously maintained laboratory cultures at Charles Sturt University, Orange, Australia (Yousuf et al. 2014b).

Choice response of the nematodes towards *Amylostereum areolatum* in the presence of *Ophiostoma ips*

In the treatment experiment, mycelial plugs (5 mm in diameter) of *A. areolatum* and *O. ips* were placed on opposite sides of a 1.5% water agar plate (4 mm thick layer of agar was used as migration matrix), 5 mm away from the edge of a 90 mm diameter Petri plate. Plates were then incubated in the dark at 20 °C for 14 h to allow the fungi to establish in the agar. The control was an uninoculated potato dextrose agar (PDA) plug placed opposite to *A. areolatum*. Nematodes were sexed (Bedding 1968) under microscope at ×20 magnification and ten female nematodes were released into the centre of the plate and allowed to move freely to feed and lay eggs. Plates were sealed with Parafilm® and placed in a dark incubator at 20 °C. Twenty replicates were used. The response of the nematodes was measured for seven days at 24 h intervals. Each day, plates were examined under a dissecting microscope and adult female nematodes within a 20 mm radius of each fungus were counted. Nematodes change their direction after encountering their host fungus and observations were made on feeding of the nematodes on the fungi. The total number of eggs laid by the nematodes on their selected fungi were also counted.

Collection of *Pinus radiata* logs

Four healthy *P. radiata* trees of similar size (approx. 15 cm DBH) and age were felled in Canobolas State Forest near Orange, New South Wales, Australia (33.3442°S, 148.9824°E). The trunk of each tree was divided into three equally sized sections and 2 m long billets (bolts) were cut from the middle of each trunk. As the tree diameter, moisture content and bark thickness changes along the length of the tree, only the middle section was used for all the experiments. *Sirex noctilio* have been observed to prefer to oviposit in the middle section of the tree (Hurley et al. 2008). The cut ends were immediately sprayed with 70% ethanol and sealed with oil paint to protect them from contamination by airborne fungi and moisture loss. The billets were transferred to a 4 °C cold room for storage until used.

Performance of *Deladenus siricidicola* in wood infected with *Ophiostoma ips* and *Amylostereum areolatum* fungi

Wood biscuits (ca. 1 cm thick and 10 cm in diameter) were cut randomly along the length of one of the aforementioned (2 m long) billets, and autoclaved at

121 °C for 30 min (Yousuf et al. 2014b). *Ophiostoma ips* and *A. areolatum* were separately grown on the wood biscuits by transferring a 5 mm diameter plug from the edge of a growing fungal culture and inoculated in the centre of the wood biscuits. *Amylostereum areolatum* was inoculated five days prior to *O. ips* due to slower growth rates (Yousuf et al. 2014b) and allowed to propagate for 15 days (enough time to infect the biscuits completely), in the dark; whereas *O. ips* required 8–10 days at 20 °C in the dark. *Amylostereum areolatum* was used as a positive control since it is known to be fed upon by *D. siricidicola*. The nematodes were extracted from *D. siricidicola* cultures as described in Yousuf et al. (2014b), washed and surface sterilised with sterile water and diluted to a final volume of 8 ml. An aliquot of 0.8 ml containing approximately 900 nematode*s* was aseptically transferred to the centre of the fungus-inoculated wood biscuits and incubated at 20 °C in the dark. There were eight replicates for each fungus treatment. Wood biscuits, fungal and nematodes cultures were used from the same source to avoid any inconsistencies in our replicates. Each biscuit was observed under a microscope at ×20 magnification after 24 h to determine the dispersal of the nematodes within the fungal matrices. The nematodes complete their life cycle from egg to adult in 4–7 days in ideal conditions (Bedding 1972). Considering this we measured population growth rate at day 7 and 21, enough to complete at least three generations. Four biscuits of each of the two treatments were destructively sampled at seven days and the other four at 21 days. The number of nematodes were counted using the Baermann funnel technique (Baermann 1917). The population growth rate (R) of the nematodes was calculated by following the method of Yousuf et al. (2014b). The difference in the growth rates were calculated between the two fungi. Numbers of eggs laid by the nematodes in *A. arealatum*- and *O. ips*-infected wood were also counted at day 7 and 21.

Nematode movement on PDA

This study determined the movement success of *D. siricidicola* through *O. ips*. Mycelial plugs (5 mm diameter) of *O. ips* and *A. areolatum* were individually placed on opposite sides of half strength PDA (prepared by dissolving 9.75 g of PDA and 2.25 g of pure agar in 500 ml of distilled water) Petri plates, containing a 4 mm thick layer of agar used as a migration matrix. *Amylostereum areolatum* was inoculated three days prior to *O. ips* due to slower growth rates (Yousuf et al. 2014a). The plate was incubated at 20 °C in the dark until both fungi occupied the plate, and the hyphae of the two species met with approximately 30% space occupied by *A. areolatum* and 70% by *O. ips*. Nematodes were extracted from *D. siricidicola* culture as described in Yousuf et al. (2014b), and approximately 100 nematodes were transferred in 0.1 ml of sterile water onto the plug of *O. ips*. There were ten replicates. The numbers of nematodes that moved toward *A. areolatum* were counted by removing *A. areolatum*-infected zone from the petri dishes. Nematodes from *A. areolatum*-infected agar were extracted using Baermann funnel technique and counted under the microscope at ×10 magnifications. Nematodes were sampled from five of the ten plates on day 2 and from the remaining five plates on day 8.

Nematode movement in wood infected with both *Ophiostoma ips* and *Amylostereum areolatum*

Twenty 15 cm long mini billets (ca. 15 cm in diameter, 431.3 g ± 13.3, mean ± SE) were cut from previously collected billets and 12 holes at equal distances were made in each billet with an increment borer. Each hole was 2 cm deep and 5 mm in diameter. Mycelial plugs of *O. ips* and *A. areolatum* were inserted alternately into the holes. *Amylostereum areolatum* was inoculated five days prior to *O. ips*. All inoculations were undertaken in a laminar flow under sterile conditions. The holes were re-sealed with the extracted wood cores and covered with masking tape. The cut ends of the mini billets were sealed with paraffin wax to retain wood moisture and prevent airborne contamination and incubated at 20 °C for 50 days or until the wood was fully occupied by the fungi. The mini billets were checked for fungal infection by taking out small 1 mm wood chips from the two extreme ends (sides) of each of the mini billets and inoculating onto PDA plates following Yousuf et al. (2014b). Subsequently, a nematode mixture, 0.8 ml (containing ca. 2000 nematodes) (Bedding 2009), was introduced into each *O. ips*-inoculation hole using a 1 ml pipette. The nematode inoculation technique used in this experiment mimicked the

industry standard pine tree inoculation technique (Carnegie and Bashford 2012) with slight modification (instead of whole tree boles, small sections (mini billets) were used). Inoculated mini billets were incubated at 23 °C in the dark. After four weeks, the *O. ips*-infected wood region was separated from the *A. areolatum*-infected wood using a chisel and hammer. Fungal-infected wood was further chopped into small chips. *Amylostereum areolatum* and *O. ips*-infected wood chips were separately soaked in tap water (200–300 ml, enough to fully soak the wood chips) and left overnight to extract the nematodes. Wood chips were removed, and the volume of the tap water was adjusted to 50 ml by allowing the nematodes to settle at the bottom of the container and carefully removing the excess water from the top. Nematodes were then mixed thoroughly, and 5 ml aliquot was used to count the nematodes. Nematodes were counted under a stereo microscope at a ×10 magnification. Total number of nematodes per fungus per mini billet was calculated by multiplying nematodes present in 5 ml with the total volume (50 ml) of water. Mean of the total number of nematodes extracted from *A. areolatum*- and *O. ips*-infected wood from all the 20 mini billets was then calculated. Population growth rate was assessed by dividing total number of nematodes recovered from initial number of nematodes inoculated (12,000) per mini billet.

Nematode movement in wood infected with sole culture of *Ophiostoma ips* or *Amylostereum areolatum*

The ends of two logs were coated with paraffin wax and 12 holes at equal distances (4 × 3 holes across the length × diameter) were made in each log with an increment borer. Each hole was 2 cm deep and 5 mm in diameter. One log was inoculated with mycelial plugs of *A. areolatum* and the other with *O. ips*. The inoculated logs were incubated for approximately two months at 23 °C in a sterile incubator. After two months 10 mini billets (15 cm in length, 8 cm in diameter, 415.5 g ± 17.9, mean ± SE) were cut from each of the two infected logs and a hole was made at 2.5 cm below the lower end of each mini billet. A nematode mixture of ca. 2000 nematodes (Bedding 2009) was then inoculated into the hole and mini billets were incubated at 23 °C. After two weeks, three, 2 cm thick wood biscuits (∼ 75 g biscuit^{-1}) were cut from each mini billet using a hand saw. The first biscuit was cut so that the nematode inoculation hole was included, and the other two biscuits taken consecutively. Each of the three biscuits per mini billet was further chopped into four pieces and soaked in the tap water (∼ 200 ml) overnight separately to extract the nematodes. The final volume of the tap water with nematodes was adjusted to 50 ml as previously describe. Samples were examined for the presence of nematodes and total number of nematodes per biscuit per mini billet was counted. Mean of the total number of nematodes present in each of the three biscuits from all the mini billets (n = 10) were calculated and reported.

Statistical analysis

Non-linear regression was applied to discriminate the significant difference in the choice response of *D. siricidicola* towards *A. areolatum* in the presence and absence of *O. ips* using the equation $y = A + B\ (R^x)$, where x is the time (in hours), y is the response of the nematodes and *A*, *B* and *R* (rate of curvature) are estimated parameters. The least square method was used because means over ten replications are considered to be approaching normality. Choice response of the nematodes towards *A. areolatum* in the presence and absence of *O. ips* was compared by Friedman ranks statistic. The effect of *O. ips* on the nematode decision was determined between the treatment and control experiments using one-way ANOVA. The differences in the number of eggs laid by the nematodes on *A. arealatum* in the two choice experiment with and without *O. ips,* were analysed using paired *t* test. The differences in the population growth of *D. siricidicola* between the two fungi, *A. areolatum* and *O. ips,* at day 7 and day 21 was analysed by one-way ANOVA. The difference in the movement of *D. siricidicola* through *O. ips* towards *A. areolatum* on PDA, on day 2 and 8, were analysed using paired *t* test. Wilcoxon rank sum tests (two-tailed) was applied to discriminate differences in the numbers of nematodes recovered from *A. areolatum*- and *O. ips*-infected wood in the nematode movement experiment. Prior to statistical analysis, the data from each experiment were checked for normality, applying Shapiro–Wilk's test. Wilcoxon rank sum test was applied when the data did not meet normality. Analyses were conducted with SPSS statistics, 17.0 (1993–2007) Polar

Engineering and Consulting (http://www.winwrap.com), GenStat 18th Edition (VSN 2015), and Microsoft Excel 2010.

Results

Choice response of the nematodes towards *Amylostereum areolatum* in the presence of *Ophiostoma ips*

The choice response of the nematodes was observed for seven days at 24 h intervals in the treatment (with *O. ips*) and control experiments (without *O. ips*). Nematodes after encountering *A. areolatum* did not change their decision. In the treatment experiment, at day 7, the nematodes (n = 20) showed a significant (df = 2; $\chi^2 = 30.62$; $p < 0.001$) preference for *A. areolatum* (6.95 ± 0.344, mean ± SE) compared with *O. ips* (0.45 ± 0.153) ($p < 0.001$) and with the nematodes that remained in the centre (2.6 ± 0.358) ($p < 0.001$).

Similar to the treatment experiment, in the control experiment at day 7, the nematodes (n = 20) also showed a significant (df = 2; $\chi^2 = 30.40$; $p < 0.001$) preference for *A. areolatum* (9. 15 ± 0.182) compared with PDA plug with no *O. ips* (0.5 ± 0.154) ($p < 0.001$) and with the nematodes that remained in the centre (0.35 ± 0.167) ($p < 0.001$).

The presence of *O. ips* affected the choice response of the nematodes. The response of the nematodes towards *A. arealatum* in the absence of *O. ips* was significantly greater ($p < 0.001$) than the response of the nematodes towards *A. arealatum* in the presence of *O. ips* (Fig. 1). At the end of the experiment (day 7), significantly ($F_{2,38} = 32.41$; $p < 0.001$) more nematodes remained in the centre and did not make any decision in the presence of *O. ips* than in the absence of *O. ips*.

Further microscopic observations showed that the nematodes fed on *A. areolatum* and established themselves. No feeding was observed on *O. ips*. The number of eggs laid (780.9 ± 91.6) on *A. areolatum* in the control experiment was significantly (df = 19; t = 2.295; p = 0.033) more than the number of eggs laid (489.3 ± 61.6) on *A. areaolatum* in the treatment experiment. No eggs were laid on *O. ips* but small numbers (4.5 ± 3.0) were laid on PDA plugs.

Performance of *Deladenus siricidicola* in wood infected with *Ophiostoma ips* and *Amylostereum areolatum* fungi

D. siricidicola was able to survive and grow on *A. areolatum* but the nematodes did not survive and grow on *O. ips*. After 24 h the nematodes dispersed all over *A. areolatum* hyphae whereas, in *O. ips,* the nematodes remained in the centre with maximum dispersal of only 10 mm.

The number of nematodes at day 7 and 21 increased significantly ($F_{1,6} = 37.926$; $p < 0.05$ and $F_{1,6} = 46.004$; $p < 0.05$) in the presence of *A. areolatum* but decreased in the presence of *O. ips*. At 21 days, the number of nematodes on *A. areolatum* (21616.7 ± 3142.6) was significantly ($F_{1,6} = 34.504$; $p = 0.001$) higher than at day 7 (3017.3 ± 387.5). However, the number of nematodes on *O. ips* (300.0 ± 41.2) at day 21 decreased significantly ($F_{1,6} = 18.307$; $p = 0.005$) compared to 7 days (604.3 ± 58.0). The growth rates of the nematodes on *A. areolatum* and *O. ips* at both day 7 and day 21 were significantly different ($F_{1,6} = 8.744$; $p = 0.025$ and $F_{1,6} = 25.541$; $p = 0.002$) (Fig. 2).

Results showed that the nematodes laid eggs in *A. areolatum*-infected wood. However, the nematodes failed to lay eggs in *O. ips*-infected wood at any time. Further analyses showed that at day 7 (5441.7 ± 1424.2), significantly ($F_{1,6} = 8.034$; $p = 0.030$) more eggs were found than at day 21 (1266.7 ± 376.1).

Nematode movement on PDA

Nematodes moved through *O. ips* towards *A. areolatum*. On day 2, 14% (n = 100) of the total inoculated nematodes moved towards *A. areolatum*, whereas, by day 8, the number of the nematodes increased significantly (df = 4; t = 5.772; p = 0.004) to 57% (n = 100). The nematodes that failed to move towards *A. areolatum* remained on the *O. ips* inoculation side. Further incubation of the plates showed that the nematodes that remained on *O. ips* moved a short distance from the inoculation site and/or did not move and died after 21 days.

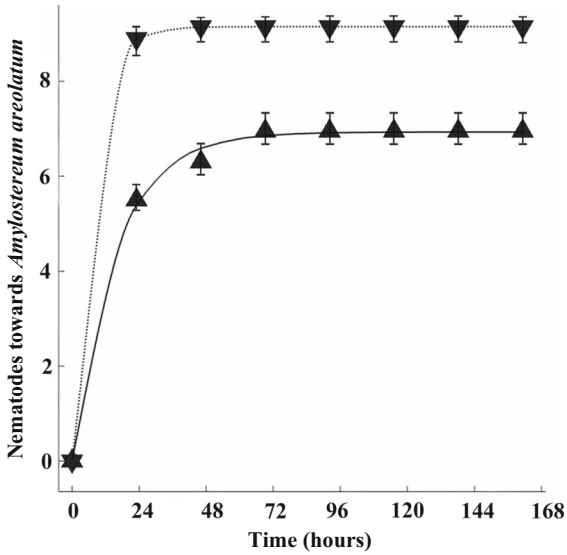

Fig. 1 Number of nematodes, *D. siricidicola,* in proximity to *Amylostereum areolatum* in the presence (*filled triangle*) and absence (*inverted filled triangle*) of *Ophiostoma ips. Points* show mean data (n = 20) and *lines* show the fitted nonlinear regression models. *Error bars* show SE. For absence of *O. ips* $Y = 9.15 - 9.15 \text{ x } (0.86^{\text{Time}})$; for presence of *O. ips* $Y = 6.93 - 6.92 \text{ x } (0.939^{\text{Time}})$

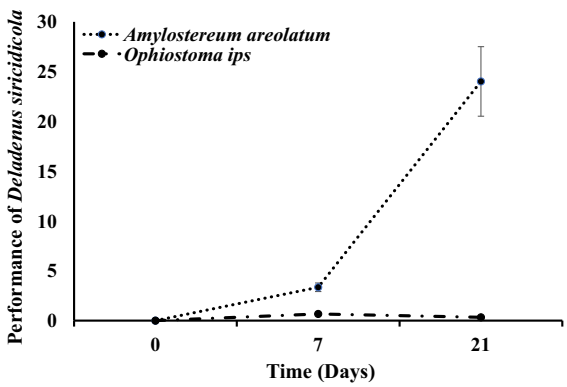

Fig. 2 Effect of diet on the growth rates of the fungal-feeding nematode, *D. siricidicola,* in wood. Growth rate of the nematodes is the mean of four replicates. *Error bars* show standard error of means

Nematode movement in wood infected with both *Ophiostoma ips* and *Amylostereum areolatum*

All the PDA plates showed active growth of the fungi from the wood chips confirming fungal infection within each of the inoculated billets. Results showed that the nematodes moved from *O. ips*-infected wood towards *A. areolatum*-infected wood. Nematodes were recovered from both fungi in all the twenty mini billets. The mean number of nematodes (n = 20) extracted from *A. areolatum*-infected wood was significantly ($df = 19$; $t = 3.91$; $p \leq 0.05$) higher ($39{,}725 \pm 6608.4$) than the mean number of nematodes extracted (3975 ± 37.4) from *O. ips*-infected wood. The growth rate (n = 20) of the nematodes per mini billet after four weeks was (3.6 ± 0.5).

Nematode movement in wood infected with sole culture of *Ophiostoma ips* or *Amylostereum areolatum*

Nematodes were present in all the three biscuits cut from *A. areolatum*-infected mini billets (n = 3 biscuits/mini billet). However, no nematodes were found from *O. ips*-infected mini billet (n = 3 biscuits/mini billet). Detailed analysis of the biscuits (first biscuit × ten replicates) cut from the mini billets (n = 10) showed that ($23{,}135.1 \pm 3997.4$) nematodes were extracted from the first biscuit, ($15{,}573.3 \pm 2402.1$) nematodes were extracted from the second biscuit cut at $\sim$ 4 cm from the inoculation hole, and ($12{,}066.7 \pm 2043.9$) nematodes were extracted from the third biscuit cut at $\sim$ 6 cm from the inoculation hole. Nematodes survived and moved within the *A. areolatum*-infected wood but failed to survive and move within *O. ips*-infected wood.

Discussion

In all experiments, *D. siricidicola* failed to reproduce or lay eggs on *O. ips.* The presence of this bark beetle-associated fungus negatively influenced the choice response of the biocontrol agent nematodes towards *A. areaolatum,* the fungus upon which it depends. The presence of *O. ips* in the wood limited the movement of *D. siricidicola* towards *A. areolatum*. These results show that the presence of *O. ips* in the trap trees may compromise biocontrol of *S. noctilio* by *D. siricidicola*.

Within 24 h of inoculation *D. siricidicola* was able to locate and established on *A. areolatum* when given a choice. There was no change in the response of the nematodes once they had made their decision. The movement of the nematodes towards *A. areolatum*

could be due to cues from esters, fatty acids or other kairomonal compounds (Balanova and Balan 1991; Jofre et al. 2016) released by *A. areolatum* (Schoonhoven et al. 2005). For example, *A. areolatum* produces volatile compounds such as acetaldehyde, ethanol, acetone and sesquiterpene 2,2,8-trimethyltricyclo [6.2.2.01,6] dodec-5-ene (Jofre et al. 2016). Female parasitoids of *I. leucospoides* use these volatile compounds to find host eggs and young larvae of *S. noctilio* within the xylem (Jofre et al. 2016). Bargmann (2006) reported that acetaldehyde is an attractant for many nematodes.

This is the first study to report a choice response in *D. siricidicola*. Other studies on the free-living nematodes *Panagrellus redivivus* (Linnaeus) (Rhabditida: Panagrolaimidae) and *M. incognita* show the primary food-finding mechanisms are governed by chemotactic factors emanating from the host or prey (Croll and Sukhdeo 1981; Balanova and Balan 1991; Perry 1996). Klink et al. (1970) showed that the fungal feeder, *Neotylenchus linfordi* (Hechler), is attracted to the secretions from fungal mycelia. The pathogenic nematode, *Pratylenchus scribneri* (Steiner) (Nematoda: Pratylenchidae), relies on chemoreception to locate its host (Bacetty et al. 2009). Other stimuli, such as thermal, vibratory, or tactile (Green 1971), are considered to play a minor role, if any, in food-finding behaviour. Results of our study show that the presence of *O. ips* negatively influenced the migratory response of the nematodes. In the presence of *O. ips*, only 69.5% moved towards *A. areolatum* whereas, in the absence of *O. ips*, 91.5% nematodes moved towards *A. areolatum.* This could be because of the production of volatile compounds (explained later in the discussion) by *O. ips*, that might have altered movement of the nematodes (Bacetty et al. 2009).

It was observed (after 24 h) that once *D. siricidicola* moved towards *A. areolatum* they did not change their decision and went on to establish on *A. areolatum.* This demonstrates that nematodes did not make their choice only after coming directly into contact with *A. areolatum* but by remotely sensed compound(s) released by the fungus. The nematodes laid eggs on *A. areolatum* and not on *O. ips.* The results from the in vivo population growth experiment show that *D. siricidicola* survived, feed, and breed in *A. areolatum*-infected wood, but failed to survive, feed or breed in *O. ips.* The results confirm the earlier reports that *A. areolatum* is the food source for *D. siricidicola* (Bedding 2009). These results are also consistent with the findings of Yousuf et al. (2014b) where the survival, growth, and reproduction of the nematodes were tested on *A. areolatum* and *O. ips* in an artificial PDA medium.

The movement of *D. siricidicola* through *O. ips* towards *A. areolatum* was affected in both PDA and wood medium. On PDA, about 43% of the nematodes failed to move through *O. ips* hyphae towards *A. areolatum* and died. However, in the wood dual culture experiment some nematodes remained in the *O. ips*-infected regions, and not all the inoculated nematodes moved towards *A. areolatum*. Studies on entomopathogenic nematodes suggest that the nematodes locate their hosts by CO_2 attraction (Triggiani and Poinar Jr. 1976), and the presence of *O. ips* might have disrupted or impaired (by releasing certain chemicals) nematodes ability to move and locate *A. areolatum*. Other studies show that some endophytic fungi produce ergot and loline alkaloids which act as repellents and may cause death of the nematodes (Bacetty et al. 2009). For example, volatile compounds produced by the fungus *Neotyphodium coenophialum* (Ascomycota: Clavicipitaceae) repel and cause mortality in the nematodes *P. scribneri* (Bacetty et al. 2009). These volatile compounds might have altered migratory behaviour of the nematodes or interfered with the chemoreception. The cause of nematodes dying on *O. ips* could be due to the production of such ergot and loline alkaloids that prevented nematode feeding and the nematodes died due to starvation.

In this study, the specific compound(s) released from the fungi were not identified or tested. However, the results support the theory of chemo-tactile behaviour by the nematodes. From this dual culture movement experiment it is not yet clear how close the host fungus needs to be for it to be detected by nematodes and for subsequent establishment. Nematodes may have died before reaching *A. areolatum* if they were inoculated far from the vicinity of their host fungus. In contrast to dual culture results, no nematodes were found alive in the wood which was solely infected with *O. ips*, whereas, in the wood solely infected with *A. areolatum,* the nematodes not only survived but also reproduced, increasing their population size. In the wood infected with both *A. areolatum* and *O. ips*, the *A. areolatum*-infected zone may have acted as a source of food which allowed the

nematodes to multiply whereas in the wood solely infected with *O. ips* the nematodes starved to death due to a lack of a food source or due to increased dryness of the wood. A recent study by Yousuf et al. (2014a) has shown that *O. ips* infection causes wood dryness. Appropriate moisture is important for nematode survival and also for the diffusion of the host cues.

In *A. areolatum*-infected wood, nematodes were widely distributed whereas in *O. ips*-infected wood no nematodes were found. This can be correlated with the release of suitable chemical signals such as esters or fatty acids, which serve as chemo-attractants (Balanova and Balan 1991) from the *A. areolatum* fungus and the olfactory/chemo-tactile behaviour of the nematodes towards its food source. In the presence of *A. areolatum* the nematode multiplied and dispersed all over the wood, continuing the free-living cycle. *Amylostereum areolatum* is a wood rot basidiomycete. Studies show that wood-rotting fungi contain two oxalate-producing enzymes—oxalo acetase and glyoxylate oxidase (Akamatsu et al. 1993; Shimada et al. 1997) which degrade the infected wood—facilitating the movement of the nematodes through the infected wood.

In the presence of *O. ips,* nematodes did not move because the fungus may have produced some alkaloids that disrupted the nematode chemoreception. Bacetty et al. (2009) have shown that alkaloids (ergot and loline) produced by a non-host endophytic fungus, *Neotyphodium coenophialum*, disrupts the chemoreception in the nematode, *P. scribneri*. A study by Zhao et al. (2013) shows that ophiostomatoid fungi produce chemical compounds e.g., diacetone alcohol (DAA). This compound increases fecundity within the nematodes. However, a specific test with *O. ips* has shown that it is the less favourable food source for the nematode *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle (Nematoda: Parasitaphelenchidae) (Zhao et al. 2013). In our results *D. siricidicola* did not feed on *O. ips* and laid no eggs. There is a possibility that *O. ips* produced DAA that may have acted as a deterrent or a non-favourable compound for *D. siricidicola*.

The larvae of *S. noctilio* use *A. areolatum* in a symbiotic relationship, as an 'external gut' for the digestion of lignocellulosic compounds resulting in a strong correlation between fungal growth inside the wood and wasp survival (Fernández Ajó et al. 2015). Consequently, the insect larvae are restricted to *A. areolatum*-infected regions in the wood (Madden and Coutts 1979; Thompson et al. 2014; Yousuf et al. 2014b). To parasitise *S. noctilio* larvae it is necessary for the nematodes to migrate to the vicinity of its insect host. So, the movement of the biocontrol nematodes towards *A. areolatum* is not only crucial for their own survival and reproduction but also obligatory to parasitise the larvae of *S. noctilio.* If the inoculation of the nematodes is done in the *O. ips*-infected region and/or far away from *A. areolatum*, there is a good chance that the nematodes may never reach *S. noctilio* larvae. They are likely to die before parasitising them. Therefore, the success of the nematode parasitism of *S. noctilio* depends directly on nematode inoculation into *A. areolatum* infected wood or near *S. noctilio* larvae.

The outcomes of this microbial study have important implications for the biocontrol of *S. noctilio* because *A. areolatum* is important to both *S. noctilio* larvae and *D. siricidicola*. This study has broadened our understanding of the interactions between the nematodes and fungi. We have demonstrated that the biocontrol nematode, *D. siricidicola,* can find and locate *A. areolatum.* But the nematode is much less able to move through *O. ips*, and can survive, establish, and multiply only if *A. areolatum* is available in close proximity. However, it is not known how far the nematodes can detect the chemical stimuli released from *A. areolatum*. Nematodes in general have both short- and long-range chemotaxis, which is widespread among different nematode taxa (Rasmann et al. 2012). It would be beneficial to understand the chemotaxis range of *D. siricidicola* as this would help to predict nematode parasitism success in trees infected with both *A. areolatum* and *O. ips.*

As *D. siricidicola* cannot survive on *O. ips*, insufficient availability of *A. areolatum* would adversely affect the rate of nematode parasitism of *S. noctilio.* This interference by *O. ips* is likely to be the cause of reduced levels of biocontrol success not only in Australia but also in other countries, such as the USA and Canada where a range of bark beetle species vector a number of different fungi into trees (Dodds et al. 2012; Ryan et al. 2012). In order to manage this problem, it is important to carefully check trap trees for infection by bark beetle-associated fungi and inoculate the nematodes only in *O. ips*- free regions in the wood as well as to take measures to manage beetle populations and their attack of trap trees.

Acknowledgements Funding for this work was provided by the Australian Research Council (ARC) Linkage Program (LP100100136) and the National Sirex Coordination Committee (NSCC). Authors would like to thank Dr. Robin Bedding for providing feedback on the manuscript. The authors would also like to thank the forestry staff from Forestry Corporation of NSW (particularly David Wright and Jo Anderson) who assisted with trap trees and billets collection and Mrs AC Johnson who assisted with manuscript preparation.

References

Akamatsu Y, Takahashi M, Shimada M (1993) Cell-free extraction of oxaloacetase from white-rot fungi, including *Coriolus versicolor*. Wood Res 79:1–6

Bacetty AA, Snook ME, Glenn AE, Noe JP, Nagabhyru P, Bacon CW (2009) Chemotaxis disruption in *Pratylenchus scribneri* by tall fescue root extracts and alkaloids. J Chem Ecol 35:844–850

Baermann G (1917) Eine einfache methode zur auffindung von ankylostomum (Nematoden) larven in erdproben. Geneeskunding Tijdschrift voor Nederlandsch-Indië 57:131–137

Balanova J, Balan J (1991) Chemotaxis-controlled search for food by the nematode *Panagrellus redivivus*. Biologia 46:257–263

Bargmann CI (2006) Chemosensation in *Caenorhabditi elegans*. WormBook

Bedding RA (1967) Parasitic and free-living cycles in entomogenous nematodes of the genus *Beddingia*. Nature 214:174–175

Bedding RA (1968) *Deladenus wilsoni* n sp. and *D. siricidicola* n sp. (Neotylenchidae) entomophagous-mycetophagous nematodes parasitic in Siricid woodwasps. Nematologica 14:515–525

Bedding RA (1972) Biology of *Deladenus siricidicola* (Neotylenchidae), an entomophagous nematode parasitic in siricid woodwasps. Nematologica 18:482–493

Bedding RA (2009) Controlling the pine-killing woodwasp, *Sirex noctilio*, with nematodes. In: Hajek AE, Glare TR, O'Callaghan M (eds) Use of microbes for control and eradication of invasive arthropods. Springer, Dordrecht, pp 213–235

Bedding R, Iede E (2005) Application of *Beddingia siricidicola* for *Sirex* woodwasp control. In: Grewal P, Ehlers R, Shapiro-Ilan D (eds) Nematodes as biocontrol agents. CABI, Wallingford, pp 385–399

Bilgrami AL, Gaugler R (2004a) Feeding bahaviours. In: Gaugler R, Bilgrami AL (eds) Nematode behaviour. CABI, Wallingford, pp 91–126

Bilgrami AL, Gaugler R (2004b) Feeding bahaviours. In: Gaugler R, Bilgrami AL (eds) Nematode behaviour. CABI, Wallingford, pp 91–126

Boender AJ, Roubos EW, van der Velde G (2011) Together or alone? Foraging strategies in *Caenorhabditis elegans*. Biol Rev 86:853–862

Carnegie AJ, Bashford R (2012) *Sirex* woodwasp in Australia: current management strategies, research and emerging issues. In: Slippers B, de Groot P, Wingfield MJ (eds) The *Sirex* woodwasp and its fungal symbiont: research and management of a worldwide invasive pest. Springer, Dordrecht, pp 175–201

Croll N, Sukhdeo M (1981) Hierarchies in nematode behavior. In: Zuckerman BM, Rohde R (eds) Plant parasitic nematodes, vol 3. Academic Press, New York, pp 227–251

Dodds KJ, Zylstra KE, Dubois GD, Hoebeke ER (2012) Arboreal insects associated with herbicide-stressed *Pinus resinosa* and *Pinus sylvestris* used as *Sirex noctilio* trap trees in New York. Environ Entomol 41:1350–1363

Fernández Ajó AA, Martínez AS, Villacide JM, Corley JC (2015) Behavioural response of the woodwasp *Sirex noctilio* to volatile emissions of its fungal symbiont. J Appl Entomol 139:654–659

Gaugler R, Lebeck L, Nakagaki B, Boush GM (1980) Orientation of the entomogenous nematode *Neoaplectana carpocapsae* to carbon dioxide. Environ Entomol 9:649–652

Gitau CW, Carnegie AJ, Nicol HI, Bashford R, Poynter C, Gurr GM (2013) Incidence of *Ips grandicollis* (Coleoptera: Scolytinae) in trap trees prepared for biological control of *Sirex noctilio* (Hymenoptera: Siricidae) in Australia: influence of environment and silviculture. For Ecol Manag 310:865–874

Green C (1971) Mating and host finding behaviour of plant nematodes. In: Zuckerman B, Mai W, Rohde R (eds) Plant parasitic nematodes, vol 2. Academic Press, New York, pp 247–266

Grewal PS (2000) Enhanced ambient storage stability of an entomopathogenic nematode through anhydrobiosis. Pest Manag Sci 56:401–406

Grewal PS, Gaugler R, Lewis EE (1993) Host recognition behavior by entomopathogenic nematodes during contact with insect gut contents. J Parasitol 79:495–503

Hurley BP, Slippers B, Croft PK, Hatting HJ, van der Linde M, Morris AR, Dyer C, Wingfield MJ (2008) Factors influencing parasitism of *Sirex noctilio* (Hymenoptera: Siricidae) by the nematode *Deladenus siricidicola* (Nematoda: Neotylenchidae) in summer rainfall areas of South Africa. Biol Control 45:450–459

Jofre N, Pildain MB, Cirigliano AM, Cabrera GM, Corley JC, Martinez AS (2016) Host selection by *Ibalia leucospoides* based on temporal variations of volatiles from the hosts' fungal symbiont. J Appl Entomol 140:736–743

Jones J (2002) Nematode sense organs. In: Lee D (ed) The biology of nematodes. Taylor & Francis Inc, New York, pp 353–368

Klink JW, Dropkin VH, Mitchell JE (1970) Studies on the host-finding mechanisms of *Neotylenchus linfordi*. J Nematol 2:106–117

Lee D (2002) Behaviour. In: Lee D (ed) The biology of nematodes. Taylor & Francis Inc, New York, pp 369–388

Madden JL, Coutts MP (1979) The role of fungi in the biology and ecology of woodwasps (Hymenoptera: Siricidae). In: Batra LR (ed) Insect–fungal symbiosis: nutrition mutualism and commensalism. Halstead Press, Sydney, pp 165–174

Murayama T, Maruyama IN (2013) Decision making in *C. elegans* chemotaxis to alkaline pH. Commun Integr Biol 6:e26633

Perry RN (1996) Chemoreception in plant parasitic nematodes. Annu Rev Phytopathol 34:181–199

Rasmann S, Ali JG, Helder J, van der Putten WH (2012) Ecology and evolution of soil nematode chemotaxis. J Chem Ecol 38:615–628

Robinson AF, Jaffee BA (1996) Repulsion of *Meloidogyne incognita* by alginate pellets containing hyphae of *Monacrosporium cionopagum*, *M. ellipsosporum*, or *Hirsutella rhossiliensis*. J Nematol 28:133–147

Rolfe R, Barrett J, Perry R (2000) Analysis of chemosensory responses of second stage juveniles of *Globodera rostochiensis* using electrophysiological techniques. Nematology 2:523–533

Ryan K, de Groot P, Davis C, Smith SM (2012) Effect of two bark neetle-vectored fungi on the on-host search and oviposition behavior of the introduced woodwasp *Sirex noctilio* (Hymenoptera: Siricidae) on *Pinus sylvestris* trees and logs. J Insect Behav 25:453–466

Schoonhoven LM, van Loon JJ, Dicke M (2005) Insect-plant biology. Oxford University Press, Oxford

Shapiro-Ilan DI, Lewis EE, Schliekelman P (2014) Aggregative group behavior in insect parasitic nematode dispersal. Int J Parasitol 44:49–54

Shimada M, Akamtsu Y, Tokimatsu T, Mii K, Hattori T (1997) Possible biochemical roles of oxalic acid as a low molecular weight compound involved in brown-rot and white-rot wood decays. J Biotechnol 53:103–113

Taylor K (1976) The introduction and establishment of insect parasitoids to control *Sirex noctilio* in Australia. Entomophaga 21:429–440

Thompson BM, Bodart J, McEwen C, Gruner DS (2014) Adaptations for symbiont-mediated external digestion in *Sirex noctilio* (Hymenoptera: Siricidae). Ann Entomol Soc Am 107:453–460

Triggiani O, Poinar GO Jr (1976) Infection of adult Lepidoptera by *Neoaplectana carpocapsae* (Nematoda). J Invertebr Pathol 27:413–414

Yousuf F, Carnegie AJ, Bashford R, Bedding RA, Nicol HI, Gurr GM (2014a) Bark beetle (*Ips grandicollis*) disruption of woodwasp (*Sirex noctilio*) biocontrol: direct and indirect mechanisms. For Ecol Manag 323:98–104

Yousuf F, Gurr GM, Carnegie AJ, Bedding RA, Bashford R, Gitau CW, Nicol HI (2014b) The bark beetle, *Ips grandicollis*, disrupts biological control of the woodwasp, *Sirex noctilio*, via fungal symbiont interactions. FEMS Microbiol Ecol 88:38–47

Zhao L, Lu M, Niu H, Fang G, Zhang S, Sun J (2013) A native fungal symbiont facilitates the prevalence and development of an invasive pathogen–native vector symbiosis. Ecology 94:2817–2826

Zuckerman BM, Jansson H (1984) Nematode chemotaxis and possible mechanisms of host/prey recognition. Annu Rev Phytopathol 22:95–113

F. Yousuf is a sessional lecturer at Charles Sturt University and a technical officer at the Orange Agricultural Institute, Department of Primary Industries. This study was part of her PhD dissertation on the interactions between the pine pests and their associated fungi and nematodes.

A. J. Carnegie is a principal research scientist with over 25 years' experience in forest health and biosecurity. He has hands-on experience in detection, diagnostics and management of pests and diseases in pine and eucalypt plantations, as well as research into impact, management, biological control and fungal taxonomy.

R. Bashford has over 50 years work experience in most aspects of forest entomology, specialising in wood borer insects and static trap monitoring systems.

H. I. Nicol is a biometrician with many years of experience in agriculture and environment. She is now working privately.

G. M. Gurr is a professor of applied ecology at Charles Sturt University in Australia. He works on applied insect ecology and developing ecologically-based strategies to combat pests.