RESEARCH ARTICLE

The bark beetle, *Ips grandicollis,* disrupts biological control of the woodwasp, *Sirex noctilio,* via fungal symbiont interactions

Fazila Yousuf[1], Geoff M. Gurr[2], Angus J. Carnegie[3], Robin A. Bedding[4], Richard Bashford[5], Catherine W. Gitau[1] & Helen I. Nicol[6]

[1]School of Agricultural & Wine Sciences, Charles Sturt University, Orange, NSW, Australia; [2]Graham Centre for Agricultural Innovation (Primary Industries, NSW and Charles Sturt University), Orange, NSW, Australia; [3]Biosecurity NSW, NSW Department of Primary Industries, Beecroft, NSW, Australia; [4]CSIRO Entomology, Canberra, ACT, Australia; [5]Forest Entomology, Forestry Tasmania, Hobart, Tas., Australia; and [6]Dalyup Statistical consulting, Orange, NSW, Australia

Correspondence: Fazila Yousuf, School of Agricultural & Wine Sciences, Charles Sturt University, 346 Leeds Parade, Orange, NSW 2800, Australia. Tel.: +61 (2) 63657652, +61 (0) 432 736 200;
fax: +61 (2) 63657578;
e-mail: fazila_yousuf@hotmail.com

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Abstract

The corticoid fungus, *Amylostereum areolatum,* is deposited in pine trees by the woodwasp, *Sirex noctilio,* at the time of oviposition. This fungus is essential in *S. noctilio* larval growth and it is also a food source for *Beddingia siricidicola,* the nematode used for *S. noctilio* biological control. In recent years, the historically successful biological control programme has been disrupted in Australia by the bark beetle, *Ips grandicollis.* This study investigated whether the mechanism of this disruption involves a fungus, *Ophiostoma ips,* which *I. grandicollis* introduces into trees. In artificial and wood media, *A. areolatum* was unable to grow in areas occupied by *O. ips.* The latter fungus was faster growing, especially at 25 °C rather than 20 °C. Larval galleries of *S. noctilio* in field-collected samples were strongly associated with wood infested by *A. areolatum* and absent from areas affected by *O. ips*. The nematode failed to survive and reproduce on *O. ips* as it can on *A. areolatum*. Competitive interactions between *O. ips* and *A. areolatum* within the trap trees are demonstrated to be key factors in the negative effect of *I. grandicollis* on *S. noctilio* biological control programmes.

Introduction

Sirex noctilio F. (*Hymenoptera*: *Siricidae*), is native to Eurasia and northern Africa (Spradbery & Kirk, 1978; Eichhorn, 1982). The wasp is an economically important pest and has caused extensive tree mortality and economic losses to the softwood industry in many exotic pine-growing countries in the Southern Hemisphere (e.g. Hurley *et al.*, 2007) and recently in several endemic *Pinus* spp. in North America (Dodds & de Groot, 2012). It is attracted to drought-stressed or damaged trees (Madden, 1977) and is also able to attack relatively healthy pine trees (Neumann & Minko, 1981; Haugen *et al.*, 1990). In Australia, it has spread to pine-growing (mainly *P. radiata*) regions in Victoria, South Australia, New South Wales and more recently inland south-eastern Queensland (Carnegie *et al.*, 2006; Carnegie & Bashford, 2012).

The corticoid fungus, *Amylostereum areolatum* (Fries) Boidin (*Basidiomycotina*: *Corticiaceace*), is an obligate symbiotic fungus of *S. noctilio* (Talbot, 1977). The fungus is deposited in pine trees by female *S. noctilio* during oviposition, along with a phytotoxic mucus which helps the fungus to grow in wood tissue causing extensive dry white rot that can kill the tree (Coutts & Dolezal, 1969; Fong & Crowden, 1973). The *S. noctilio–A. areolatum* complex is also essential for larval nutrition, egg eclosion (by creating a suitable environment) and contributes to larger adult insect size and reproductive success (Madden & Coutts, 1979; Madden, 1981).

A parasitic nematode, *Beddingia* (= *Deladenus*) *siricidicola* (*Nematoda*: *Sphaerulariidae*), is used for the biological control of *S. noctilio* (Bedding, 1984). The adult female nematode is extraordinary in having two distinct morphological forms: a free-living (mycophagous) form that feeds and reproduces on *A. areolatum* and a parasitic (entomophagous) form that sterilizes the female *S. noctilio* (Bedding, 1972). The biological control programme includes trap tree plots (with *c.* 10 trees) that

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are treated with a weak rate of herbicide to stress trees, so making them attractive to ovipositing *S. noctilio*. Nematode masses reared on *A. areolatum* (Bedding & Akhurst, 1974) are then inoculated in these trap trees. These nematodes then feed on *A. areolatum*, reproduce and disperse within each tree to locate and parasitize the *S. noctilio* larvae, resulting in sterilized female *S. noctilio* carrying nonviable eggs filled with juvenile nematodes. These eggs are then oviposited in nearby trees, leading to effective local control of the *S. noctilio* population. *Beddingia siricidicola* has been released in every Southern Hemisphere country where *S. noctilio* is a pest and is generally recognized as the main controlling agent (Bedding, 2009). In Australia, nematode control has generally been successful, with the ability to cause almost 100% parasitism (Bedding & Akhurst, 1974; Neumann & Minko, 1981).

This biological control programme in Australia is now in jeopardy due to the unprecedented interference by an exotic bark beetle, *Ips grandicollis* Eichhoff (*Coleoptera*: *Scolytidae*). This beetle attacks trap trees and is suspected to be associated with a decrease in parasitism in recent years (Carnegie & Loch, 2010; Carnegie & Bashford, 2012). It is native to North and Central America and attacks stressed, weakened or damaged trees and slash (Erbilgin *et al.*, 2002) but can attack healthy trees if insect density is high.

Recent surveys indicate that attack on *P. radiata* trap trees by *I. grandicollis* is common and is associated with low (7–44%) nematode parasitism rates of *S. noctilio* (Carnegie & Loch, 2010). The mechanism/s by which this interference operates is unknown and needs to be understood to protect the efficacy of biological control of the potentially devastating *S. noctilio* woodwasp. The interaction could be multipartite where plant–insect–microbial associates might be involved in causing detrimental effect on each other. *Ips grandicollis* vectors a grey-staining fungus, *Ophiostoma ips* (Rumbold) Nannfeldt (*Ophiostomatales*: *Ophiostomataceae*) (Hunt, 1956; Smalley *et al.*, 1993), into the wood. This fungus has been reported as being vectored by other species of bark beetles (e.g. Zhou *et al.*, 2001). *Ophiostoma ips* plays an important role in tree mortality (Mathre, 1964) by colonizing parenchyma rays, resin ducts and tracheids and causing blockage which results in weakened timber and aesthetically undesirable staining (Kopper *et al.*, 2004). Given that *O. ips* utilizes the same wood substrate as *A. areolatum,* there is scope for these two fungi to competitively interact when present in the same tree. The outcomes of this microbial competition could have important implications for biological control of *S. noctilio* because *A. areolatum* is important for both *S. noctilio* larvae and *B. siricidicola*. These interactions among plant, insects and their fungal associates will help us in understanding such complex system inside the same host plant.

No direct research has been conducted on these interactions or their implications. Accordingly, this study tested the following aims: (1) the nature of the interactions between *A. areolatum* and *O. ips* in artificial and wood media; (2) the impact of temperature on these interactions; (3) the effect of differing lengths of time between inoculations of the initial and then the second fungus; (4) the effect of these fungal interactions on *S. noctilio*; and (5) studies on the response of *B. siricidicola* to *O. ips*.

Material and methods

Fungal and nematode isolation

Amylostereum areolatum was directly isolated from adult female *S. noctilio,* reared from infested *P. radiata* billets that were obtained from a site located in a *P. radiata* plantation in Carabost State Forest, near Tumut (35.37.9° S, 47.45.9° E), New South Wales (NSW), Australia. *Ophiostoma ips* was isolated from the *I. grandicollis*-infested *P. radiata* bark, collected from Canobolas State Forest, near Orange, in NSW (33.3442° S, 148.9824° E), in April 2011.

Fungal colonies were provisionally identified based on microscopic observations of hyphal morphology and melanization. The identities of *A. areolatum* and *O. ips* were further confirmed by the NSW Department of Primary Industries diagnostic laboratory through sequencing of internal transcribed spacer regions 1 and 2 between the ribosomal RNA genes *18S* and *28S*. Vouchers of both isolates (*A. areolatum*-DAR 82117 and *O. ips*-DAR 82118) were lodged at the Agricultural and Scientific Collections Unit, Department of Primary Industries, Orange, NSW.

Beddingia siricidicola nematodes were isolated directly from the testes of male *S. noctilio* (obtained from rearing billets) and cultured on one-third-grown *A. areolatum* plates. More detailed information about fungal and nematode isolation and culturing is given in the Supporting Information, Data S1 (Supplementary Material S1).

Sole culture of *O. ips* and *A. areolatum in vitro* and in wood

An initial experiment examined the growth of both fungi in sole culture conditions on artificial medium [potato dextrose agar (PDA)] and on *P. radiata* wood biscuits at two different temperatures (20 and 25 °C) to ascertain growth rates of each fungus in the absence of the other. Detailed information on the method is given in the Data S1, Supplementary Material (S2).

Dual culture interactions between *O. ips* and *A. areolatum* in *vitro*

Interactions between *O. ips* and *A. areolatum* were studied on PDA. There were two replicates. In the first replicate, two temperatures were applied in two incubators. In the second replicate, the temperatures were switched between incubators. In the first main treatment, *A. areolatum* plates were inoculated 0, 3, 6 and 9 days after the inoculation of the same plate with *O. ips.* Fungi were reversed in the second main treatment. There were six replicates of each treatment combinations per incubator. Mycelial plugs (5 mm diameter) of *O. ips* and *A. areolatum* were plated on opposite sides of each plate, 65 ± 5 mm away from each other. Plates containing a single plug of each species were used as a control. All the plates were fully randomized within each incubator. This experiment was designed to elucidate the effect of different inoculation timing of one fungus on the growth of the other.

Plates were inspected daily until both fungi reached equilibrium (mycelia of both species meeting after which there was no further growth of either fungus). Thereafter, plates were observed every 10 days for up to 50 days to give full opportunity for either fungus to overgrow the other. The hyphae of each fungus were of different colours, and hence, colony boundaries were determined visually using a dissecting microscope. At the end of the experiment, photographs were taken using a Canon D60 camera. The colony area of each fungus was measured from the photographs using IMAGE J software (U. S. National Institutes of Health, Bethesda, MD).

The viability of each species of fungus after the dual culture experiment was assessed to determine any detrimental effect of either of the fungi on the other. Two plates from each treatment and temperature were randomly selected and two mycelial plugs from the hyphal meeting point were transferred to the centre of the new PDA plates. One plug was transferred to each new plate. In addition, two mycelial plugs were also taken from each colony, 10 mm away from the hyphal meeting point of the two fungi. Four replicates were made for each treatment and temperature. All plates were incubated for 10 days at 20 °C and were then observed for fungal growth. The observations were based on the active growth of each fungus inoculated on new PDA.

Dual culture interactions between *O. ips* and *A. areolatum* in wood

Wood biscuits were soaked in sterile distilled water and autoclaved twice at 121 °C for 30 min. Biscuits were then used as the medium for a dual culture experiment similar to that described above for PDA plates where both fungi were inoculated simultaneously. Biscuits were inoculated as in the *in vitro* study. Each biscuit was placed on a glass rod (to prevent the filter paper from sticking), which was set in a Petri plate on moist, sterilized filter paper (following Ryan *et al.*, 2011) that maintained an 80% moisture level within the biscuits. Petri dishes with biscuits were sealed with Parafilm and placed singly in a row inside the incubator at 20 and 25 °C. Five replicates were used for each temperature. The two temperatures were randomly allocated to the two incubators and randomly reallocated for the second replication. Fungal colonies were measured for radial growth every day or until colonies reached equilibrium. Colony boundaries on biscuits were easy to distinguish visually, as they had distinctive hyphal appearance. Photographs were taken and colony area of each fungus was calculated.

To check the viability of both fungi in the wood medium, fungal re-isolation was carried out by taking a wood chip from each hyphal meeting point and 10 mm away from the fungal hyphal meeting point with a sterilized, sharp chisel. Each wood chip was transferred to a new PDA plate. Plates were incubated at 20 °C for 10 days, and observations were based on the active growth of each fungus inoculated on new PDA. Five biscuits from each temperature treatment were used after 10 days of fungal inoculations, and the remaining five biscuits were used after 60 days of fungal growth.

Interactions between *O. ips* and *A. areolatum* within the wood of trap trees

Interactions between *O. ips* and *A. areolatum* were studied in the trap trees (Detailed information on trap trees collection is given in Data S1, Supplemental Material S3). Briefly, field observations allowed trees to be categorized into (1) those attacked by *I. grandicollis* first and later by *S. noctilio*; (2) those attacked by both species simultaneously; and (3) those attacked by *I. grandicollis* after *S. noctilio.* Biscuits were cut from trees and the area affected by *O. ips* was calculated. Detailed information about the method is given in Data S1 Supplementary Material S4.

Effect of fungal interactions in trees on *S. noctilio*

The biscuits used previously in the fungal study (section 2.5) were further analysed for the presence of *S. noctilio* larval galleries. The numbers of individual *S. noctilio* larval galleries were counted from each biscuit. The analyses were made from a total of 900 biscuits from the trap trees ($n = 15$) attacked by both insects simultaneously that also

had both fungi present. Data were recorded for the presence and absence of larval galleries in white- and grey-stained areas of the wood biscuits. The number of larval galleries per biscuit was counted, and a scatter plot was drawn for the number of *S. noctilio* larval galleries against percentages of *O. ips* staining.

Performance of *B. siricidicola* on *O. ips* and *A. areolatum*

Cultures of *O. ips* and *A. areolatum* were prepared on PDA (full and quarter strength, see Data S1, Supplementary Material S5) as described previously. Each fungus was allowed to grow until it covered 1/3 of the plate at 20 °C, with 30 replicates (10 plates per increment of 7, 14 and 21 days) of each fungus. *Amylostereum areolatum* was considered a positive control as it is known to be fed upon by *B. siricidicola*. About 1800 surface-sterilized *B. siricidicola* were aseptically transferred to the centre of the fungus-inoculated plates, and 10 plates were placed inside the incubators running at 20 and 25 °C. At 7, 14 and 21 day increments, nematodes were counted using the Baermann funnel technique (Baermann, 1917) in ten of the plates for each temperature. Population growth rate (R) of the nematodes was calculated as follows: R = pf/pi, where, pf is the number of nematodes extracted on (7, 14 and 21) days and pi is the number of inoculated individuals per plate (*c.* 1800). The procedure was then repeated on one further occasion, with temperatures allocated to incubators in a reversed manner, to the first occasion, to give two temporal replicates. Plates were allocated randomly within incubators.

Statistical analyses

The influence of *A. areolatum* and *O. ips* on each other *in vitro* and in wood studies was analysed by measuring the colony radius and area at each assessment time with factorial and split-plot analysis of variance (ANOVA). Separate two-way ANOVA was used to compare the effect of temperature and fungus, when inoculated in dual culture simultaneously on PDA and wood. Binomial distribution with a logit link function was fitted to test for any inhibitory effect of one fungus on the other at the hyphal meeting point or away from the hyphal meeting point of the two fungi as per chi-squared statistics. The differences in the growth and reproduction of *B. siricidicola* at two temperatures were analysed by a split-plot ANOVA with temperatures as main plots. Fungal viability tests both in *vitro* and in wood were analysed by a chi-squared test. The effect of fungal interactions in trees is shown graphically.

Analyses were conducted with GenStat 15th Edition (VSN 2012) and SPSS statistics, 17.0 (1993–2007) Polar Engineering and Consulting (http://www.winwrap.com/). Graphs were generated on SIGMAPLOT version 11.0 (Systat Software, Inc.).

Results

Sole culture of *O. ips* and *A. areolatum in vitro* and in wood

Comparisons of the radial growth of *O. ips* and *A. areolatum* grown separately showed marked differences in growth on PDA. *Ophiostoma ips* grew significantly more rapidly than did *A. areolatum* at both temperatures, 20 °C ($d.f._{1,88}$, $F = 16.212$, $P < 0.0001$) and 25 °C ($d.f._{1,88}$, $F = 60.385$, $P < 0.0001$; Fig. S1a). The higher temperature significantly ($d.f._{1,68}$, $F = 4.956$, $P < 0.05$) promoted growth of *O. ips*; an effect that was less marked for *A. areolatum* (Fig. S1a).

In the wood medium also, *O. ips* showed significantly faster growth than *A. areolatum* at 20 °C ($d.f._{1,88}$, $F = 5.553$, $P < 0.05$) and 25 °C ($d.f._{1,88}$, $F = 10.638$, $P < 0.005$; Fig. S1b). There was no significant difference between temperatures in the radial growth of *A. areolatum* ($d.f._{1,88}$, $F = 0.200$, $P > 0.05$) in the wood. However, *O. ips* exhibited significantly faster radial growth at the higher temperature ($d.f._{1,88}$, $F = 1.642$, $P < 0.05$; Fig. S1b).

Dual culture interactions between *O. ips* and *A. areolatum* in *vitro*

At 20 °C, *O. ips* grew significantly faster than *A. areolatum* when two fungi (inoculated simultaneously, time zero) were compared under dual culture conditions ($d.f._{1,214}$, $F = 6.656$, $P < 0.05$; Fig. 1a). Growth of both *O. ips* and *A. areolatum* in dual culture did not differ significantly from the growth of their respective isolates in sole culture for the first 8 days ($d.f._{1,166}$ $F = 1.821$ $P > 0.05$ and $d.f._{1,214}$, $F = 0.029$, $P > 0.05$, respectively), but thereafter, growth in dual culture slowed significantly compared to sole cultures as the colonies of *O. ips* and *A. areolatum* in dual culture reached the edge of the other fungal species colony ($d.f._{1,46}$, $F = 253.521$, $P < 0.0001$ and $d.f._{1,46}$, $F = 73.167$, $P < 0.0001$, respectively; Fig. 1a).

At 25 °C, *O. ips* grew significantly faster than *A. areolatum* when two fungi were compared under dual culture conditions (inoculated simultaneously–time zero; $d.f._{1,214}$, $F = 114.646$, $P < 0.0001$; Fig. 1b). Growth of *O. ips* and *A. areolatum* in dual culture did not differ significantly for the growth in sole culture for the first 6 days ($d.f._{1,118}$, $F = 3.153$, $P > 0.05$ and $d.f._{1,118}$, $F = 0.573$, $P > 0.05$, respectively), but thereafter, growth slowed

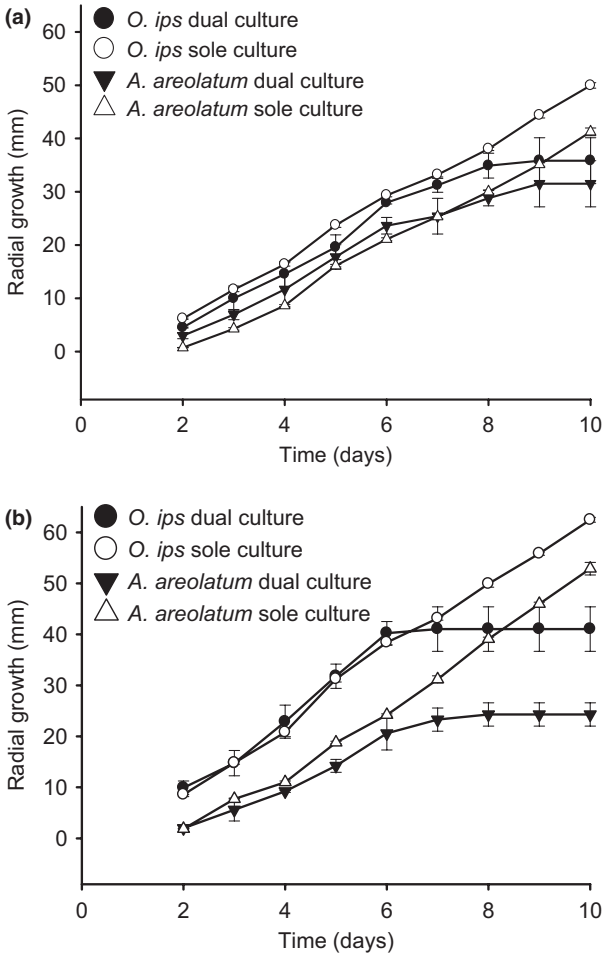

Fig. 1. Cumulative radial growth (mean ± SE) of *Ophiostoma ips* and *Amylostereum areolatum* together in dual culture (n =12) on PDA medium at (a, 20 °C; and b, 25 °C).

significantly as the colonies of *O. ips* and *A. areolatum* in dual culture reached the edge of the other colony (d.f.$_{1,94}$, $F = 118.748$, $P < 0.0001$ and d.f.$_{1,94}$, $F = 215.927$, $P < 0.0001$; Fig. 1b).

There was a significant difference between the two temperatures in the extent of radial growth of *A. areolatum* (d.f.$_{1,214}$, $F = 7.839$, $P < 0.05$) and *O. ips* (d.f.$_{1,214}$, $F = 23.501$, $P < 0.0001$) in dual culture (inoculated simultaneously–time zero; Fig. 1a and b). High temperature (25 °C) favoured *O. ips* growth.

There was a marked effect of the duration of prior inoculation time (3, 6 and 9 days) on the extent of radial growth of the second fungus (d.f.$_{3,176}$, $F = 3327.46$, $P < 0.001$; Fig. S2a and b). In dual culture conditions, *A. areolatum* occupied progressively more plate area as the time delay of *O. ips* inoculation increased (d.f.$_{2,72}$, $F = 1353.632$, $P < 0.0001$; Fig. S2a). Similarly, inoculating *A. areolatum* 3, 6 and 9 days after *O. ips* in dual culture disadvantaged *A. areolatum* to a progressively greater extent as the time of prior inoculation increased (d.f.$_{2,72}$, $F = 3794.071$, $P < 0.0001$; Fig. S2b).

Temperature had a significant effect on the area occupied by both fungi with different time delays (3, 6 and 9) (d.f.$_{3,176}$, $F = 52.61$, $P < 0.001$) and with prior inoculations of either fungi (d.f.$_{1,176}$ $F = 14.13$ $P < 0.001$). In all time delay experiments in dual culture, *A. areolatum* showed better growth at lower temperature of 20 than 25 °C (d.f.$_{3,96}$, $F = 5.966$, $P < 0.001$; Fig S2a); (d.f.$_{3,96}$, $F = 42.192$, $P < 0.0001$; Fig. S2b).

Neither species was able to occupy space already colonized by the other species. When the hyphae of both fungi met, only limited intermingling growth occurred and no subsequent expansion occurred for either fungus over the course of 10 days. *Amylostereum areolatum* hyphae tended to grow only 2–3 mm above the *O. ips* colony, while *O. ips* growth extended below *A. areolatum* for 1–1.5 mm. Production of fruiting bodies (perithecia of *O. ips* and spores of *A. areolatum*) appeared to be stimulated along the zone of hyphal contact. Ongoing monitoring for 60 days after fungal hyphae met revealed no further changes in hyphal intermingling growth although *O. ips* produced many perithecia below its zone of intersection with *A. areolatum*.

The fungal viability results showed that at hyphal meeting points, *O. ips* was active and *A. areolatum* was active in all except for one treatment, where it was inoculated 9 days after *O. ips*. The probability of *A. areolatum* being active in the presence of *O. ips* was $P = 0.922$. There was no effect of temperature on the activeness of either fungus. Both fungi were active away from the hyphal meeting point when taken from their respective growth region $P = 1$. This result showed that there was no toxic effect of the fungus on each other.

Dual culture interactions between *O. ips* and *A. areolatum* in wood

Ophiostoma ips grew significantly faster than *A. areolatum* when the two fungi were compared under dual culture conditions at 20 °C (d.f.$_{1,98}$, $F = 51.628$, $P < 0.0001$; Fig. S3a). Growth of *O. ips* in dual culture was significantly greater than growth in its sole culture (d.f.$_{1,98}$, $F = 8.461$, $P < 0.005$); however, growth of *A. areolatum* in dual culture did not differ significantly from the growth in sole culture (d.f.$_{1,98}$, $F = 0.220$, $P > 0.05$).

At 25 °C, *O. ips* grew significantly faster than *A. areolatum* when two fungi were compared under dual culture conditions (d.f.$_{1,98}$, $F = 76.129$, $P < 0.0001$; Fig. S3b). Growth of *O. ips* was significantly greater than growth in sole culture (d.f.$_{1,98}$, $F = 25.462$, $P < 0.05$). Growth of *A. areolatum* did not differ significantly for the growth in

sole culture for the first 4 days ($d.f._{1,58}$, $F = 0.899$, $P > 0.05$), but thereafter, growth was significantly greater in sole culture than dual culture ($d.f._{1,38}$, $F = 26.142$, $P < 0.0001$; Fig. S3b).

Further analysis for another 60 days showed no further colony expansion, but the wood previously occupied by *O. ips* changed to a darker grey stain, characteristic of this fungus.

Samples of both fungi taken after dual culture were consistently viable in re-isolations from the hyphal meeting point and away from the hyphal meeting point, and there was no toxic effect of either fungus on the other when tested after 10 days. In the latter case, when tested after 60 days of incubation, *O. ips* showed more active growth than did *A. areolatum*. There was a significant difference in the active growth of both fungi at two timings (10 and 60 days; $d.f._{1,32}$, $F = 7.2$, $P = 0.011$), where the active growth of both fungi reduces with time.

Interactions between *O. ips* and *A. areolatum* within wood of trap trees

Biscuits cut from trap trees that were infested with *I. grandicollis* first ($n = 900$) had an average area of 96.44% (SE 0.114) occupied by *O. ips* (e.g. Fig. 2a). Biscuits from trees that were infested with *I. grandicollis* and *S. noctilio* simultaneously ($n = 900$) had a mean proportion of 49.99% (SE 0.855) occupied by *O. ips* (e.g. Fig. 2b).The corresponding figure for biscuits from trap trees that were infested with *S. noctilio* first and later by *I. grandicollis* ($n = 300$) was 0.84% (SE 0.088) (e.g. Fig. 2c). In all cases, the entire area of the wood samples was fully utilized by one or other fungus but neither overgrew the other; neither was there any indication of an inhibition or exclusion zone inside the wood. All 15 samples taken from the grey-stained region yielded *O. ips* growth; however, only 11 of 15 samples taken from the white-stained region yielded *A. areolatum*. Adventitious fungi interfered with the isolation of both fungi, and this could be the reason why fewer samples from white-stained regions yielded *A. areolatum*.

Effect of fungal interactions in trees on *S. noctilio*

Galleries of *S. noctilio* were conspicuous within the wood samples but present only in wood occupied by *A. areolatum* (Fig. 2). No galleries were present in the wood occupied by *O. ips* (Fig. 2). Up to three galleries were recorded in any one wood biscuit, with the mean number greatest for samples from trees with low levels of *O. ips* (Fig. 3).

Performance of *B. siricidicola* on *O. ips* and *A. areolatum*

Numbers of *B. siricidicola* increased in the presence of *A. areolatum* (Fig. 4a), but markedly decreased in the presence of *O. ips* ($d.f._{1,18}$, $F = 109.124$, $P < 0.0001$; Fig. 4b). The nematodes failed to lay eggs in the presence of *O. ips,* and no feeding activity was observed at any time and temperature. Temperature was a significant factor for *B. siricidicola* performance on both fungal diets. Nematodes increased more rapidly at 20 °C than at 25 °C on *A. areolatum*. There was a significant difference between the two temperatures in the population growth rate of nematodes at 14 days ($d.f._{1,18}$, $F = 127.559$, $P < 0.0001$) and 21 days ($d.f._{1,18}$, $F = 37.679$, $P < 0.0001$). However, no significant difference was found between the two temperatures at 7 days ($d.f._{1,18}$, $F = 4.100$, $P = 0.058$) on *A. areolatum* (Fig. 4a). On *O. ips,* nematodes decreased more rapidly at 25 °C. There was a significant difference between the two temperatures at the following days (7 days = $d.f._{1,18}$,

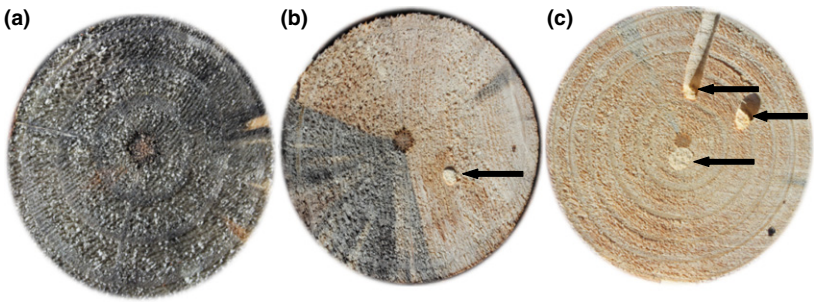

Fig. 2. Wood biscuits cut from *Ips grandicollis*- and *Sirex noctilio*-infested trees showing the growth pattern of *Ophiostoma ips* (grey staining) and *Amylostereum areolatum* (white staining) and the presence of *S. noctilio* larval galleries (marked with black arrows). Biscuit from tree infested with *I. grandicollis* prior to *S. noctilio* (a), biscuit from tree infested with *I. grandicollis* and *S. noctilio* simultaneously (b) and biscuit from tree infested with *S. noctilio* prior to *I. grandicollis* (c).

$F = 23.485$, $P < 0.0001$), (14 days = $d.f._{1,18}$, $F = 7.790$, $P < 0.05$) and (21 days = $d.f._{1,18}$, $F = 32.430$, $P < 0.0001$) on *O. ips* (Fig. 4b).

Discussion

This study tested the possible causes for the inconsistent biological control of *S. noctilio* by *B. siricidicola* observed recently in Australia. Fungal interactions between *A. areolatum* and *O. ips* and the effect on the larval growth of *S. noctilio* and on the survival of *B. siricidicola* were examined. The results supported the hypothesis that *I. grandicollis* is responsible for the interference in the biological control of *S. noctilio* by introducing its associated fungus *O. ips* into the trap trees.

Ophiostoma ips was found to grow faster than *A. areolatum* in artificial and in wood media. In dual culture, when both fungi were inoculated simultaneously, *O. ips* colonized more substrate than *A. areolatum* and subsequently prevented *A. areolatum* from colonizing the space. Despite differences in radial growth, *A. areolatum* showed strong defence capabilities and was never replaced by *O. ips*. This differs from the findings of King (1966), where *A. areolatum* was always overtaken by competitors (commonly occurring wood-inhabiting fungi, such as *Trichoderma* sp. and *Diplodia pinea* (Desm.) Kickx.), but similar to the findings of Ryan *et al.* (2011) and Hurley *et al.* (2012) who worked with *O. minus* (Hedgcock) Sydow et *P.* Sydow and *O. ips,* respectively.

The different inoculation timings of either fungus showed that there was a marked effect of the duration of prior inoculation time on the extent of radial growth of the second fungus. *Amylostereum areolatum* succeeds in occupying more space in dual culture conditions with time delays only when inoculated prior to *O. ips*. However, *A. areolatum* was not able to occupy more surface area in dual culture conditions when inoculated after *O. ips* due to the differences in the growth rate of each fungus. Possible interpretation of these results is that if *I. grandicollis* attacks the trap trees prior to *S. noctilio* then it spreads *O. ips* which grows rapidly and limits the space available for the late-arriving *A. areolatum*. Alternatively, if *A. areolatum* arrives first, then it can occupy more space than *O. ips*.

Results from fungal interaction in wood showed similar outcomes as *in vitro* studies. In all interactions, no antagonistic or stationary barrier or zone between the two interacting fungi was observed, which differs from the

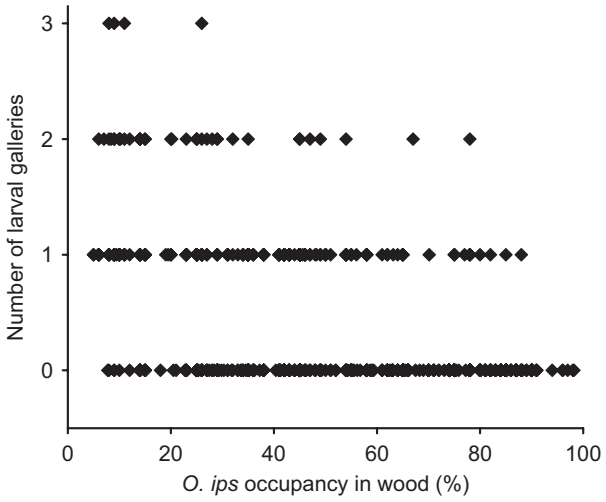

Fig. 3. Effect of *Ophiostoma ips* on number of *Sirex noctilio* larval galleries present in wood biscuits. Total biscuits ($n = 900$), from 15 trap trees that had *Amylostereum areolatum* and *O. ips*.

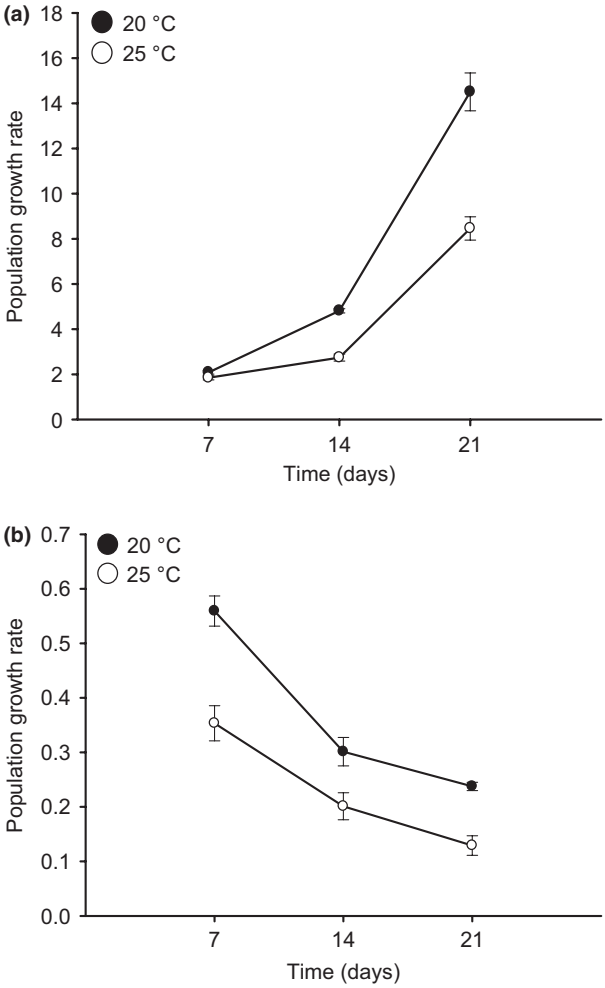

Fig. 4. Effect of diet and temperature on *in vitro* population of the fungal-feeding nematode, *Beddingia siricidicola*, (a, *Amylostereum Areolatum;* and b, *Ophiostoma ips*). Population growth rate is the mean of $n = 10$.

results of Hurley *et al.* (2012). This difference could be due to the use of a different strain of *O. ips* or *A. areolatum* or to the different medium used. The *O. ips* used by Hurley *et al.* (2012) was isolated from galleries of different bark beetle, *Orthotomicus erosus* (Wollaston), and the *A. areolatum* strain was isolated from laboratory cultures of *B. siricidicola* sent from Australia in 2003. In our study, *O. ips* was isolated from galleries of *I. grandicollis,* and *A. areolatum* was directly isolated from female *S. noctilio*. The use of different media could also be a factor in the different outcomes of these studies; we used PDA and wood, while malt extract agar was used in the study by Hurley *et al.* (2012). Both *A. areolatum* and *O. ips* showed intermingling growth for a few millimetres before reaching stabilization or inhibition of growth, which was previously described by Boddy (2000) and Skidmore & Dickinson (1976). Although the mechanisms of inhibition of growth of the tested fungi were not addressed in our study, the significant inhibition in growth at the hyphal contact point of both fungi could be due to the production of inhibitory substances or could be due to the competition for the nutrients or both (Nourozian *et al.*, 2006; Ryan *et al.*, 2011).

We also found that temperature plays an important role in the growth of *A. areolatum* and *O. ips.* High temperature (25 °C) increased the growth of *O. ips* but hindered *A. areolatum* growth, whereas lower temperature (20 °C) favoured *A. areolatum* growth. The attack of *S. noctilio* raises the internal temperature of *P. radiata* 1−5 °C above the air temperature by affecting its transpiration as the tree dies (Jamieson, 1957), resulting in further competitive pressure on the *A. areolatum* after its initial establishment in a tree and improving conditions for the growth of *O. ips*.

The fungal interaction observed in the billets obtained from trap trees infested with *I. grandicollis* and *S. noctilio* clearly supports the competitive nature of *O. ips.* Under natural conditions, *O. ips* colonized more wood than *A. areolatum* within trap trees that were either attacked by *I. grandicollis* or simultaneously attacked by *S. noctilio* and *I. grandicollis*, whereas trees that were attacked by *S. noctilio* first had a lower percentage of *O. ips. Amylostereum areolatum* is a slow-growing fungus (King, 1966) and was able to spread itself freely only in the absence of other fungi.

Amylostereum areolatum is necessary for *S. noctilio* egg eclosion and for the survival of *S. noctilio* larvae, which exclusively feed on *A. areolatum* before entering the wood (Madden & Coutts, 1979). In this study, we found that no larval galleries were present in the region of *O. ips*-infected wood. However, larval galleries were present in the *A. areolatum* region, suggesting that *S. noctilio* seem to be confined to wood free of *O. ips*. It is clear from our results that the presence of *O. ips* correlates with the absence of larval tunnels. Lack of larval galleries in the *O. ips* region could be due to the possibility that eggs of *S. noctilio* are not hatched in the presence of *O. ips* or the larvae starve to death due to unavailability of food. There could also be a possibility that *O. ips* may repel ovipositing *S. noctilio*, resulting in no larval galleries in biscuits. The latter aspect was studied by Ryan *et al.* (2012) where the oviposition activity of female *S. noctilio* was tested against two blue-staining fungi, *Leptographium wingfieldii* Morelet and *O. minus*, vectored by *Tomicus piniperda* (L.) bark beetle attacking *Pinus* species. They reported that *S. noctilio* can detect blue-staining fungi and does not oviposit. However, it is not known whether *S. noctilio* rejects the whole tree or searches for an *O. ips*-free patch in the same tree to oviposit. If *S. noctilio* rejects the whole *O. ips*-infected trap tree, then it might oviposit in other nearby suitable trees (other than trap trees) where there is no biocontrol nematode. The result being an increase in unparasitized wasps emerging slowing the biological control programme.

The biocontrol nematodes, *B. siricidicola*, grow and reproduce only on *A. areolatum. Beddingia siricidicola* does not feed or lay eggs on *O. ips*. A temperature of 25 °C decreased the growth rate of *B. siricidicola*, whereas it increased the growth of *O. ips*. Consequently, at this temperature, the increased growth rate of *O. ips* inside the wood would result in limiting food resources for the biocontrol nematodes and that is likely to contribute to low levels of parasitism. This may also result in the emergence of unparasitized *S. noctilio* from trap trees that are infested with *I. grandicollis* prior to *S. noctilio*. The response of nematodes to temperatures is important to consider as global climate change could affect the biocontrol of *S. noctilio* due to the direct effect of temperature on *A. areolatum* by favouring *O. ips* development.

Sirex noctilio larvae are present only in *A. areolatum* regions in trees infested with *O. ips.* So it is important to check the regions in a tree with *O. ips* when inoculating biocontrol nematodes, because if nematodes are inoculated in *O. ips* region, then *O. ips* can restrict nematode migration resulting in unparasitized *S. noctilio*. Hence, defined areas of a trap tree that are free of *O. ips* should be considered for current and future biological control. Trap trees should be examined prior to inoculation for heavy grey staining. If the trees are heavily stained, then they should be discarded as unsuitable for nematode inoculation.

The growth and competitive ability of the fungi examined in this study offer important insights into factors that might affect the establishment of *A. areolatum* in the field and consequently the survival of *B. siricidicola*. Our work is the first to show quantitative evidence of the outcomes of interactions between the *A. areolatum* and

O. ips under laboratory and natural conditions. In our study, we have examined the influence of temperature on the outcomes of interactions which is one of the key factors in climate change. In this study, we have shown for the first time that the biocontrol nematode agent is adversely affected by the presence of *O. ips*. This information may be helpful for understanding the cause of reduced levels of biocontrol success not only in Australia but also in other countries such as North America and Canada where there are many native bark beetles that vector a number of different fungi into trees.

Further research is needed to understand and minimize the problem of *S. noctilio* biological control inconsistency. For example, it is important to understand how *I. grandicollis* and *O. ips* affect the growth and survival of *S. noctilio* larvae within the wood, whether the larvae continue to tunnel when they come into contact with *O. ips* or change their path or stop and die due to starvation. There is an opportunity to develop strategies that can minimize *I. grandicollis* attack or prevent *O. ips* from spreading into trap trees. For example, if *A. areolatum* is inoculated into the trap trees during tree poisoning, then *A. areolatum* has more time and opportunity to spread itself freely in the absence of contending fungi such as *O. ips*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Comparison of the cumulative radial growth (mean ± SE) of *Ophiostoma ips* and *Amylostereum areolatum* (n = 10) in sole culture (a, PDA and b, wood).

Fig. S2. Percentage of surface area occupied (mean ± SE) by *Amylostereum areolatum* in the presence of *Ophiostoma ips* at day delay (0, 3, 6 and 9) in a dual culture experiment at 20 and 25 °C (a, *A. areolatum* was inoculated first and b, *A. areolatum* was inoculated after *Ophiostoma ips*).

Fig. S3. Cumulative radial growth (mean ± SE) of *Ophiostoma ips* and *A. areolatum* together in dual culture (n = 10) in wood medium at (a, 20 °C and b, 25 °C).

Supplementary Material (S1–S5).