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Performance of *Sirex noctilio*'s biocontrol agent *Deladenus siricidicola*, in known and predicted hosts

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HIGHLIGHTS

- Growth of Sirex fungus was lower on hybrid pine substrate than on *Pinus taeda*.
- Survival of Sirex biocontrol nematodes was lower in hybrid pine than *P. taeda*.
- Plant chemistry may contribute to differences in fungus and nematode performance.

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ABSTRACT

Survival of the free-living mycetophagous form of *Deladenus siricidicola*, the major biological control agent of Sirex woodwasp, *Sirex noctilio*, was tested in known (*Pinus taeda*) and predicted novel (*P. elliottii* subsp. *elliottii* × *P. caribaea* var. *hondurensis*) hybrid host taxa. Trials were established in the field to simulate nematode dispersal both naturally by infected wasps and following commercial inoculation, as well as in the laboratory under controlled conditions. Nematodes showed reduced survival in hybrid pine compared with *P. taeda* for all tree-associated treatments, but performed equivalently in petri-dish bioassays containing substrate of each taxon. Growth of *Amylostereum areolatum*, the food source of *D. siricidicola* was lower on plates containing ground hybrid substrate than on plates containing ground *P. taeda*. Some physical differences were found between taxa, including differences in bordered pit diameters, tracheid widths, and basic density, but these did not consistently explain reduced performance. More plant secondary compounds (predominantly oleoresins) were present in hybrid taxa than in *P. taeda*, and in standing trees compared with felled trees. Our results suggested that *D. siricidicola* may not be as effective in hybrid pine taxa for the biological control of *S. noctilio* as it is in its current known host taxa, possibly because of reduced growth of its food source, *A. areolatum* in hybrid pine.

1. Introduction

Sirex woodwasp, *Sirex noctilio* F. (Hymenoptera: Siricidae) and its obligate symbiotic fungus, *Amylostereum areolatum* (Chaillet ex Fr.) Boidin (Russulales: Amylostereaceae) are major invasive pests of softwoods worldwide. In concert, the duo kills trees through the combined action of the wasp's phytotoxic mucus and growth of the fungus, both deposited into trees during oviposition (Ryan and Hurley, 2012). The two species are established as exotic invaders in Australia, New Zealand, South Africa, China, South America and North America (Adams et al., 2011; Li et al., 2015), and have gradually expanded their Australian geographic range, reaching southern (temperate) Queensland in 2009 (Carnegie and Bashford, 2012). Climate modelling predicts subtropical regions of Queensland will support *S. noctilio* establishment (Carnegie et al., 2006), suggesting the wasp is likely to continue to move north into valuable subtropical pine estates, where previously unencountered hosts, particularly southern pines (*Pinus elliottii* (Engelm) var. *elliottii* and *P. caribaea* var. *hondurensis* (Sénécl.) W.H. Barrett and Golfari) (Pinales: Pinaceae) and their hybrids, are grown in large-scale plantations (Gavran, 2014). No *Pinus* species are known to be resistant to Sirex, although the level of susceptibility varies between species (Ryan and Hurley, 2012): it is likely that these hybrids will be suitable hosts but uncertain how host characteristics may interact with biological control (Bedding, 2009).

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The parasitic nematode, *Deladenus* (=*Beddingia*) *siricidicola* Bedding (Tylenchida: Neotylenchidae), is the most effective biological control agent used in *S. noctilio* management worldwide, but shows very variable and inconsistent parasitism rates within and between regions (Hurley et al., 2007). Mechanisms behind the variation in biological control success using nematodes have not been clearly explained (Hurley et al., 2008; Slippers et al., 2012). Extremely low genetic diversity of *D. siricidicola* may affect their ability to adapt to novel environments and hosts (Mlonyeni et al., 2011), and aspects of the host trees themselves may also affect nematode survival (Bedding, 2009). The efficacy of the standard biological control program developed in winter rainfall regions appears to be reduced in different climatic conditions, as was the case in South America and South Africa (Slippers et al., 2012). Thus, there are concerns that if the woodwasp establishes in the subtropical pine estate in Australia, the current biological control practice may not be effective.

Deladenus siricidicola has a free-living fungal-feeding form that is mass-produced for commercial inoculation in Sirex biological control programs (Slippers et al., 2012). In proximity to Sirex larvae, female nematodes shift phase to a parasitic form that ultimately sterilises female Sirex by entering the ovaries and eggs, and is then transmitted (as the free-living, fungal-feeding form) during oviposition by the infected wasp (Slippers et al., 2012). The free-living form is thus spread naturally when infected *S. noctilio* females lay packets of nematodes in place of viable eggs into trees, and operationally by inoculating laboratory-cultured nematodes into felled trees. The success of the biological control program is influenced by interactions between wasp, nematodes and fungal strains (Morris et al., 2012; Slippers et al., 2015), other insects and fungi (Yousuf et al., 2014), climatic conditions (Hurley et al., 2007) and host tree characteristics (Bedding, 2009).

Loblolly pine, *Pinus taeda* L., native to SE USA, is grown extensively in South America and, along with *P. elliottii* is the most commonly planted commercial pine species in Brazil and Argentina (Klasmer and Botto, 2012) and the most susceptible to *S. noctilio* (Fenili et al., 2000; Iede et al., 2012). In this host, *S. noctilio* parasitism by *D. siricidicola* varies between 0 and 90% (Iede et al., 2012; Nahrung et al., 2015). *Pinus taeda* is grown commercially in southern Queensland and northern New South Wales, where it is readily attacked by *S. noctilio* (Carnegie et al., 2005), albeit less successfully than *P. radiata* (Nahrung et al., 2015). A commercial hybrid *Pinus* taxon, *Pinus elliottii* subsp. *elliottii* x *P. caribbea* var. *hondurensis*) (PEE × PCH) was developed in 1958 (Nikles, 1996) and is now widely grown in coastal plantation regions of south-eastern Queensland. The performance of Sirex wasp, fungus and nematode in this host is unknown, and hence the overall impact of the Sirex wasp in this host is impossible to predict. Here, we use field and laboratory studies, in the absence of *S. noctilio*, to compare performance and survival of both the *Amylostereum* fungus and the free-living mycetophagous form of *D. siricidicola* on these different host taxa. We also compare physical and chemical properties of the two hosts with potential to impact on the survival and development of nematodes and/or fungus, including moisture content, wood density, tracheid and bordered pit diameter.

2. Materials and methods

2.1. Nematode survival and development

2.1.1. Field trials

Field trials were established in a subtropical plantation (Beerburrum, Site 1, PEE × PCH F2 hybrids) and a higher altitude, temperate plantation (Passchendaele, Site 2, *P. taeda*) (Fig. 1) in Queensland, and intended to mimic (a) natural nematode spread by ovipositing females (i.e. into standing trees, n = 5/site) and (b) commercial inoculation into felled trees (n = 5/site). Because *S. noctilio* is not yet present in the subtropics (Site 1), surrogate techniques were used to introduce *A. amylostereum* and *D. siricidicola* into all trees. All trees were poisoned using standard trap tree plot techniques designed to stress trees and make them susceptible to *S. noctilio* oviposition (Haugen et al., 1990; Gitau et al., 2013) to provide conditions similar to those that nematodes would encounter either deposited by Sirex females (standing trees), or during artificial inoculation (felled trees). Ten trees at each site were poisoned with Dicamba (3,6-dichloro-2-methoxybenzoic acid) at a rate of 1 mL/10 cm circumference in March 2012. In May 2012, five trees at each site were felled, and five were left standing. Each tree was inoculated at four points (approximately 0, 90, 180 and 270 degrees around the stem) at each 1 m interval along the trunk for 4 m (i.e. 16 inoculation points per tree) with a slurry of nematode rearing media containing both nematodes and the *Amylostereum* fungus supplied by Ecogrow Environment Ltd. Nematodes were mixed to a concentration of approximately 1 million nematodes/500 mL of Amylostereum rearing slurry, and inoculated into the tree using the standard hammer punch method (Carnegie and Bashford, 2012), such that each inoculation point received approximately 995 nematodes. Samples were taken by removing a 4 cm diameter core around the inoculation point to a depth of 1 cm with a hole saw at each inoculation height (1–4 m) one and three months after inoculation. The outer bark was removed to minimise inclusion of any bark feeding nematodes, and then cores were soaked in 30 mL of tap water for 12 h and the supernatant examined under a dissecting microscope (40×) for the presence of nematodes. The proportion of cores per tree that scored positive for nematodes was calculated, and results for each sample time and treatment were compared using chi-square tests.

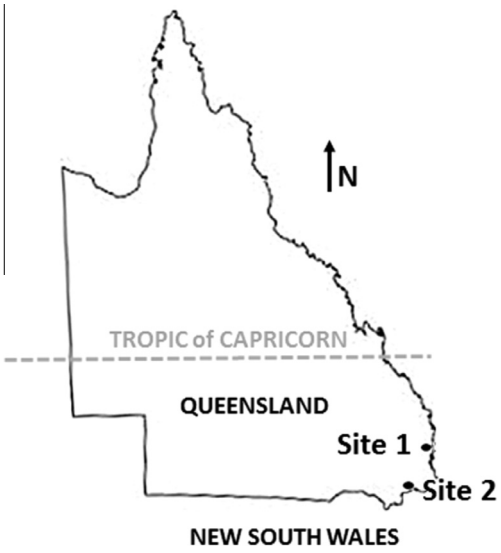

Fig. 1. Map of Queensland, Australia, showing the location of the two field sites. Site 1 (Beerburrum) comprises hybrid pine grown in the subtropics; Site 2 (Passchendaele) comprises *Pinus taeda* grown in higher altitude, temperate conditions.

2.1.2. Laboratory trials

To enable comparison between taxa under identical conditions, three inoculated billets from the top of each felled tree from the field trial above were taken to the laboratory immediately following inoculation, and stored in a controlled environment room (20 °C, 60% RH). One billet from each tree was destructively

sampled at one and three months post-inoculation, by cutting into slices 3.5 ± 1.5 cm thick, and soaking each in 300 mL of water separately in a plastic bag for 12 h. The number of nematodes in each sample was estimated by decanting the supernatant into a flat dish and allowing nematodes to settle to the bottom. The number of nematodes in each of four fields of view of a dissecting microscope (40×) was counted and the total number of nematodes per sample was estimated by correcting for the proportion of the total surface area that our counts represented. We also examined the distribution of nematodes relative to the inoculation point by combining data from slices to the equivalent of approximately 10 cm intervals, and mapping the proportion of nematodes recovered from each section.

2.2. Amylostereum survival and spread

Growth of *Amylostereum* fungus on an artificial substrate incorporating each of the two taxa was compared. Outer sapwood samples were collected from the same trees as the field trials by removing cross-sections between 4 and 10 cm thick about 2 m from the tree base. The bark was removed from each biscuit and sections of outer sapwood chosen to minimise blue-stain fungus. Samples were ground in a rotary mill (Siemens) with a 1 mm mesh, then sieved to separate and collect all material between 0.5 and 2 mm (coarse), and less than 0.5 mm (fine). Approximately equal amounts of the coarse ($0.503 \pm 9.9 \times 10^{-4}$ g) and fine ($0.502 \pm 1.0 \times 10^{-3}$ g) ground sapwood samples from each tree were mixed together, sterilised by irradiation (25 kGy for 25 h), mixed with agar (1.2%, Oxoid Technical Agar #3, 15 mL) and poured into separate Petri dishes. Control plates (agar only) were also prepared.

Amylostereum areolatum used in the trials was sourced from a commercial culture maintained by Ecogrow Environment Pty Ltd. The Ecogrow culture was subcultured onto Potato Dextrose Agar (PDA, 3.9%, Difco, 15 mL) plates and grown for use in the trials. The prepared agar plates containing ground sapwood were inoculated with a plug (3.3 mm diameter) of the *A. areolatum* culture placed on the surface of the agar in the centre of each plate. Plates were sealed with Parafilm™ and stored in the dark at 26 °C for the duration of the experiment.

Three measures of fungal growth were made for each plate. Radial growth was measured from the central inoculation point to the furthest point of hyphal growth visible under a dissecting microscope (40×) (mean of four readings from 0, 90, 180 and 270°). Overall hyphal density was a subjective measure of density of hyphal growth across the plate (scale of 0–3). Hyphal expansion was a subjective measure of the density of hyphae at the leading edge of the radial expansion (scale of 1–6). Assessments were made at 4, 7, 11 and 14 days post inoculation, except hyphal density and hyphal expansion which began on Day 7. By Day 14 hyphal growth had reached the edge of the petri dish in most cases, preventing accurate measures of hyphal density. Data were analysed using Mann-Whitney U-tests, adjusted for multiple comparisons using a Bonferroni correction.

Half of the plates used for assessing fungal growth were inoculated with nematodes eight days after initial inoculation with *Amylostereum*. A 10 μL suspension of nematodes from Ecogrow (1 in 24 dilution) was inoculated at three positions on each plate (0, 120 and 240°) giving approximately 60 nematodes per plate. Plates were sealed with Parafilm™ and incubated at 23 °C in darkness.

After twelve days, plates were visually assessed under a dissecting microscope for the presence (low/medium/high) or absence of nematodes and given a score from 0 to 3. Plates were then stored at 4 °C until final assessment based on Morris et al. (2012). Briefly, plates were filled with 15 mL of distilled water and allowed to sit for 10 min then swirled and the supernatant was poured off. Plates were then rinsed four times and all washings were combined. The number of nematodes present (viewed under a dissecting microscope at 25×) was assigned to 'high', 'medium' and 'low' categories, and given a score from 1 to 3.

2.3. Physical properties

2.3.1. Moisture content and wood density

Moisture content was measured at inoculation using the wood plug removed for sampling and at three months using the core removed for nematode sampling. Samples were transported cooled, to the laboratory in sealed airtight bags, then weighed prior to soaking for nematode extraction (wet mass). After soaking, samples were dried at 103 °C, then reweighed until they reached constant mass (dry mass). Moisture content was then estimated as ((wet mass-dry mass)/(wet mass)). These data were arcsine-square root transformed prior to analysis using a two-sample *t*-test. Basic wood density was estimated using the equation (dry mass/wet volume) using cross-sectional slices of mid-bole billets stored under controlled conditions. These data were compared using a Mann-Whitney *U*-test because of non-homogeneity of variance.

2.3.2. Tracheid and bordered pit diameter

Test billets were cut 2 m from the base of the standing trees used in inoculation trials (n = 3 per taxon). A 4 cm thick disc was cut from the lower end of each billet, and a 15 mm diametral strip was cut from each disc through the "pith" from edge-to-edge (bark removed). Particular attention was paid to avoiding any obvious defects in the sample, such as compression wood, resin pockets and branch knots. The study looked at the 2nd and 5th growth rings from the bark, to approximately mimic the hammer inoculation depth and ovipositor penetration range of Sirex females. The 2nd and 5th ring segments were extracted from the best radii of the sample and dissected into separate earlywood and latewood material. Each separated sample was tangentially cut into "matchstick" sized pieces and placed in individual 30 mL test tubes ensuring sample integrity throughout. Thus, for each taxon, there were replicated samples from early and late wood for each of ring 2 and ring 5 for each of three trees (n = 12 samples per taxon).

Samples were macerated by adding 10 mL of freshly prepared macerating fluid (1:1 mixture of hydrogen peroxide 100 vols – 35% W/W and glacial acetic acid) to each 30 mL test tube with vented cap and placing test tubes into 500 mL beakers containing 250 mL gently simmering water. The beakers were placed on top of a heating plate in a fume cabinet and simmered gently for 5 h (keeping water in the beakers at an appropriate level) by which time the samples had reached a totally colourless state. The test tubes were removed from the beakers and the macerating fluid was carefully poured off, taking care not to loosen fibres in the process. The samples were washed several times with deionised water to remove all trace of the macerating fluid and allowed to cool before storage at 4 °C. Temporary slides were made in order to capture digital images of individual fibres. Samples were shaken vigorously to separate fibres and a drop of the mixture was slide mounted using Safranin stain (1% in 95% alcohol diluted 50/50 with deionised water). Six slides for each of the 12 samples were prepared for digital photography using a "Leica" DMLB compound microscope with digital image capture capability coupled with a NIKON "Digital Sight" using DS-L2 Version 322 software. Each slide was viewed at 40× magnification and visually scanned, in a systematic manner to locate suitable pits for image capture. Sufficient slides were scanned and images captured to ensure at least 50 pits were included. The required measurements were obtained using *Image-Pro Plus V6.3* digital imaging software.

Pit apertures on the top plane of the fibre were measured, initially along the longest diameter and then at 90° to the initial diameter. As many of the suitable pit apertures as possible were measured to ensure adequate co-efficient of variation. Three internal fibre diameters were measured, at the point where the pit apertures were measured and averaged to give the "mean fibre width". Data were analysed using one-way ANOVA, with Fisher's protected LSD post hoc tests.

2.4. Chemical properties

Further samples of ground sapwood used in the petri dish bioassays were taken, and approximately equal amounts of the coarse ($1.004 \pm 1.0 \times 10^{-3}$ g) and fine ($1.004 \pm 1.3 \times 10^{-3}$ g) samples were mixed together and extracted in dichloromehane (Ajax, 10 mL) for 48 h in the dark at 4 °C (after Cadahia et al., 1997; Eyles et al., 2003; Hayes et al., 2014). Samples were stored in the freezer until analysis.

Samples (1 μL) were analysed using a gas chromatograph (GC) (Agilent 6890 Series) coupled to a mass spectrometer (MS) (Agilent 5975) and fitted with a silica capillary column (Agilent, model HP5-MS, 30 m × 250 μm ID × 0.25 μm film thickness). Data were acquired under the following GC conditions – inlet temperature: 250 °C, carrier gas: helium at 51 cm/s, split ratio 13:1, transferline temperature: 280 °C, initial temperature: 40 °C, initial time: 2 min, rate: 10 °C/min, final temperature: 260 °C, final time: 6 min. The MS was held at 280 °C in the ion source with a scan rate of 4.45 scans/s.

Peaks present in blank dichloromethane (control) samples were discarded from analysis in test samples. Tentative identities were assigned to peaks with respect to the National Institute of Standards and Technology (NIST) mass spectral library. Mass spectra of peaks from different samples with the same retention time were compared to ensure that the compounds were indeed the same.

Mann-Whitney U-tests were used to compare the number of compounds between treatments, and ANOSIM was used to determine differences in overall chemical profiles, with post hoc *t*-tests used to determine whether relative peak areas differed significantly for compounds found to separate the groups.

3. Results

3.1. Field trials

3.1.1. standing trees

No *D. siricidicola* were recovered from any cores from any standing hybrid trees at either of the sample times (n = 20 cores/sample). Nematodes were recovered from all *P. taeda* trees, from an average of 45 ± s.e. 9.3% of cores/tree at the one-month sample, and from 60% of trees and 12.5 ± 7.2% of cores/tree at three months post-inoculation. Taxa were significantly different at the one-month sample ($\chi^2_1 = 11.6$, $P < 0.001$), but not after three months ($\chi^2_1 = 3.2$, $P = 0.07$).

3.1.2. Felled trees

One month after inoculation, nematodes were recovered from all felled *P. taeda* trees, and three-quarters of cores/tree (75 ± 16%), but only from three (60%) hybrid trees, and less than half of cores sampled (40 ± 17%) ($\chi^2_1 = 5.01$, $P = 0.03$). After three months, however, nematodes were recovered similarly from both taxa, at 30 ± 0.15% of cores from *P. taeda*, and 30 ± 0.12% cores from hybrid pine ($\chi^2_1 = 0.01$, $P = 0.09$).

3.2. Laboratory trials

3.2.1. Whole tree sections

After one month, there was a greater than sevenfold difference in the number of nematodes recovered from *P. taeda* billets than hybrid billets stored under controlled conditions since inoculation. On average, each ten-centimetre section of *P. taeda* had 17,341 ± 4591 estimated nematodes, significantly more than hybrids, which had only 2248 ± 557 per section (Mann-Whitney *U*-test, U = 25, P = 0.008). On examining the distribution of nematodes throughout the billet, there was a significant, negative relationship between the number of nematodes and the distance from the inoculation point for *P. taeda* (Spearman rank correlation, $\rho = -0.65$, $P < 0.001$), but not for hybrids ($\rho = -0.13$, $P = 0.25$), with nematodes appearing normally distributed around the inoculation point for *P. taeda*, but not for hybrids (Fig. 2).

Three months after inoculation, although *P. taeda* supported over five times as many nematodes as hybrids (*P. taeda*: 1770 ± 1504; hybrid 322 ± 228), the average estimated number of nematodes per section was not significantly different between the taxa (Mann-Whitney *U*-test, U = 19, P = 0.222).

3.2.2. Petri dish bioassays

Amylostereum areolatum grew on both ground-wood + agar substrates (Fig. 3), but not on agar-only control plates. The radial growth (mm) of *A. areolatum* did not differ between felled and standing trees within each taxon (Mann-Whitney U tests, U = 1.5–6.0, P = 0.04–1.0; Bonferroni-adjusted P = 0.02), or between *P. taeda* and hybrid pine (Mann-Whitney *U* test, U = 23–30, P = 0.38–0.88) (Fig. 3).

However, hyphal density (Fig. 4: Mann-Whitney *U* test, U = 9–13.5, P = 0.01–0.04) and hyphal expansion (density at the leading edge of fungal growth) (Fig. 5: Mann-Whitney *U* test, U = 4.5–11, P = 0.003–0.03) were always higher in *P. taeda* than the hybrid, and both were always higher than the plain agar control, which scored 0 and 1, respectively, at each assessment.

Twelve days after plates were inoculated with nematodes, nematode eggs were present on 7/8 hybrid-substrate plates, and 4/8 *P. taeda* substrate plates; this difference was not statistically different ($\chi^2_1 = 2.6$, $P = 0.11$). Likewise, the number (high, medium or low) of nematodes did not differ between plates of different taxa (Mann-Whitney *U* test, U = 21.0, P = 0.27), with hybrid plates scoring an average 2.13 ± 0.13 and *P. taeda* scoring 1.75 ± 0.16. At the

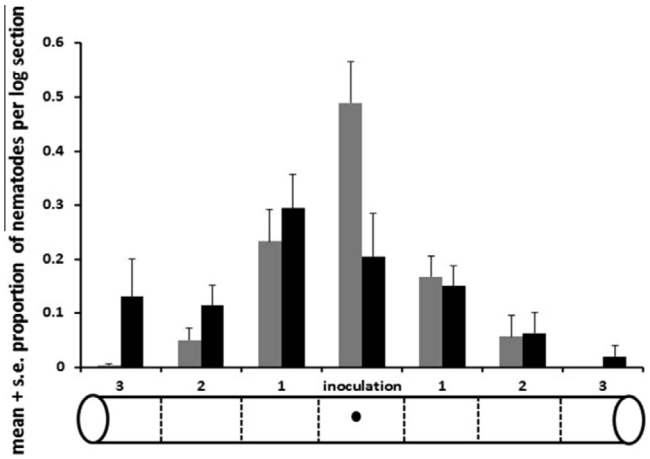

Fig. 2. Mean + s.e. proportion of nematodes recovered from each 10 cm section of *Pinus taeda* (grey) and hybrid pine (black) billets kept under controlled conditions, for one month after inoculation into the centre of the billet.

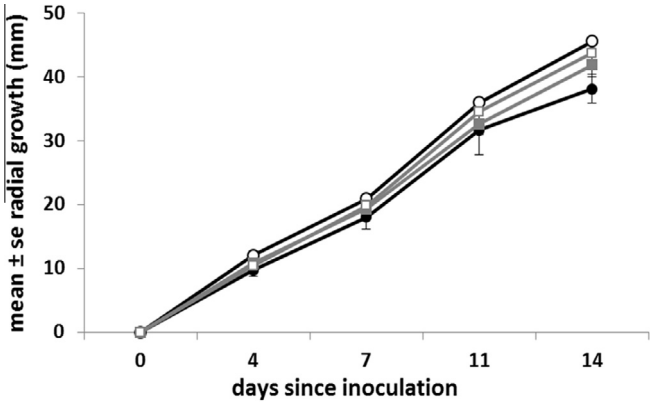

Fig. 3. Mean ± s.e. radial growth (mm) of *Amylostereum areolatum* on agar plates containing either felled (open symbols) or standing (solid symbols) ground *Pinus taeda* (squares) or hybrid pine (circles).

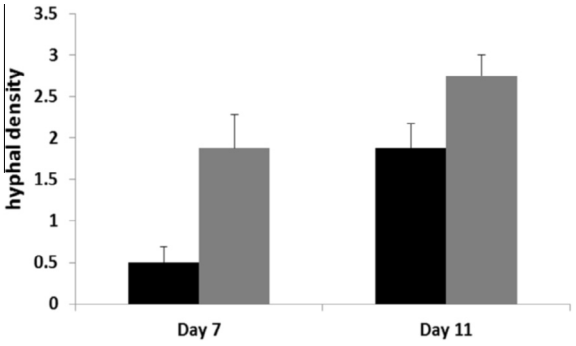

Fig. 4. Hyphal density of *Amylostererum areolatum* measured seven and eleven days after inoculation onto agar plates containing ground *P. taeda* (grey) and hybrid (black). Hyphal density differed between taxa at both assessments (see text).

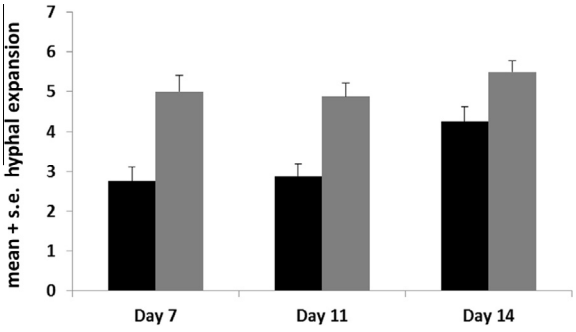

Fig. 5. Hyphal expansion (density at the leading edge of fungal growth) of *Amylostererum areolatum* measured seven, eleven and fourteen days after inoculation onto agar plates containing ground *P. taeda* (grey) and hybrid (black). Hyphal expansion differed between taxa at each assessment (see text).

conclusion of the experiment, nematode populations persisting on both taxa were not statistically separable (U = 17.5, P = 0.17), with an average score of 2.13 ± 0.30 for hybrid plates, and 1.38 ± 0.32 per *P. taeda* plate.

3.3. Physical properties

3.3.1. Moisture content and wood density

Initial moisture content, and moisture content three months after inoculation did not differ between taxa for any treatment (Table 1), however the basic density of hybrid wood

Table 1
Mean ± s.e. moisture content of *Pinus taeda* and hybrid pine treatments at inoculation (i), and three months after inoculation (3) with *Deladenus siricidicola* and *Amylostereum areolatum*.

	Field-standing	Field-felled	Laboratory
Pinus taeda (i)	0.57 ± 0.01	0.58 ± 0.02	0.62 ± 0.01
Hybrid (i)	0.55 ± 0.01	0.57 ± 0.02	0.61 ± 0.01
t-test	t_8 = 1.86	t_8 = 0.1	t_8 = 0.31
	P = 0.1	P = 0.92	P = 0.76
Pinus taeda (3)	0.45 ± 0.01	0.31 ± 0.2	0.33 ± 0.02
Hybrid (3)	0.39 ± 0.02	0.30 ± 0.03	0.34 ± 0.02
t-test	t_8 = 10.26	t_8 = 0.06	t_8 = 0.46
	P = 0.05	P = 0.95	P = 0.66

(0.037 ± 0.001 g/m^3) was significantly greater than that of *P. taeda* (0.034 ± 0.001 g/m^3) (Mann-Whitney *U* test, U = 1403, P = 0.004).

3.3.2. Tracheid and bordered pit diameter

Overall, pit diameter was larger in hybrids than in *P. taeda* (*t*-test, t_{22} = 4.7, P < 0.001). Considering samples separately, however, the difference was significant only in ring 5 (ANOVA, $F_{7,23}$ = 3.4, P = 0.02, Fishers' protected LSD post hoc test) (Fig. 6).

Tracheid width also differed between taxa, this time being larger in *P. taeda* than in hybrids but only in ring 2 earlywood (ANOVA, $F_{7,23}$ = 3.9, P = 0.01, Fisher's protected LSD post hoc test) (Fig. 7).

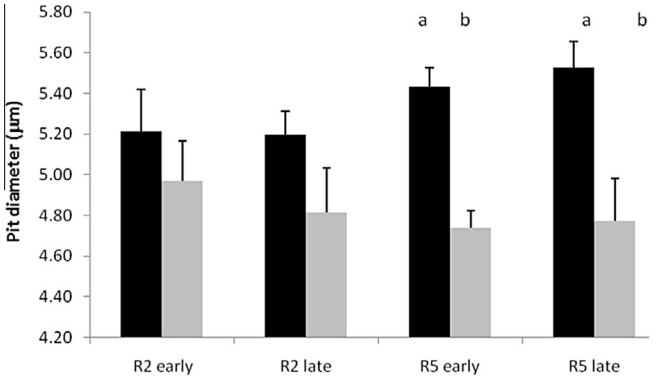

Fig. 6. Average + s.e. pit diameter (µm) from growth ring 2 (R2) and 5 (R5) early and late wood of *Pinus taeda* (grey) and F2 hybrids (black). Different letters above bars designate means that differ significantly.

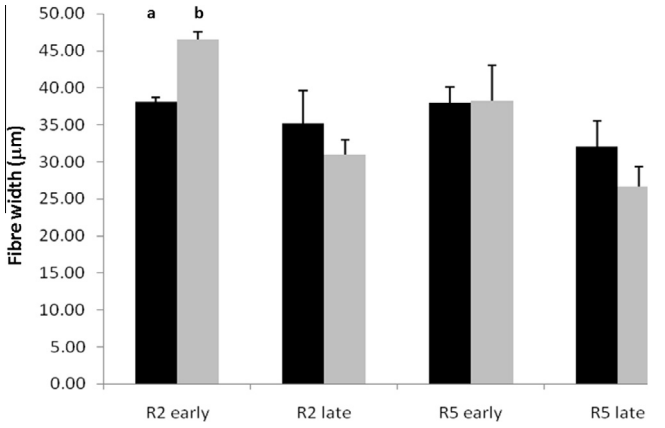

Fig. 7. Average + s.e. tracheid width (µm) from growth ring 2 (R2) and 5 (R5) early and late wood of *Pinus taeda* (grey) and F2 hybrids (black).

3.4. Chemical analysis

For each taxon considered separately, there were significantly more compounds in standing than felled trees (*P. taeda*: U = 0.0, P = 0.036; hybrid: U = 0.0, P = 0.036), and significantly more in standing trees when both taxa were combined (Mann-Whitney U = 0.0, P < 0.001). However, there was no difference between the taxa with respect to number of compounds for either standing (U = 1.5, P = 0.400) or felled trees (U = 5.5, P = 0.175). Standing hybrids contained three unique compounds, and standing *P. taeda* had one unique compound (Fig. 8). Overall, seven compounds were detected in hybrids that were not found in *P. taeda* (Fig. 6) and two in *P. taeda* but not in hybrid.

Considering all trees, there was no difference between the taxa in their total chemical profile (ANOSIM: Global R = 0.08, P = 0.135. However standing trees differed from felled trees (Global R = 0.268, P = 0.047) and so were considered separately. The taxa differed for standing trees only, although the groups overlapped (Global R = 0.481, P = 0.10), with significantly more palustic acid, tolurosol and an unidentified compound (RT 20.233, MW 286) detected in hybrids than in *P. taeda* (Table 2). For felled trees, the two taxa were barely separable (Global R = 0.208, P = 0.143), with relative amounts of callistic acid, dehydroabietic acid (DHA) and methyl 4-hydroxy-2-methoxy-3,5,6-trimethyl benzoate contributing most strongly to group separation. For standing trees, seven compounds contributed to the top 50% of dissimilarity between taxa – of which 71% had retention times greater than 20 min (i.e. non-volatile compounds). For felled trees, 8 compounds contribute to the top 50% of dissimilarity – of which 63% had retention times greater than 20 min (Table 3).

Of the total 88 compounds detected during the analysis less than one third had retention times greater than 20 min (ie non-volatile compounds), although they were 60–70% of the compounds that differed between taxa. Oleoresins such as these present both a physical and chemical defence system against stem-boring insects and are composed mostly of monoterpenes and diterpene resin acids.

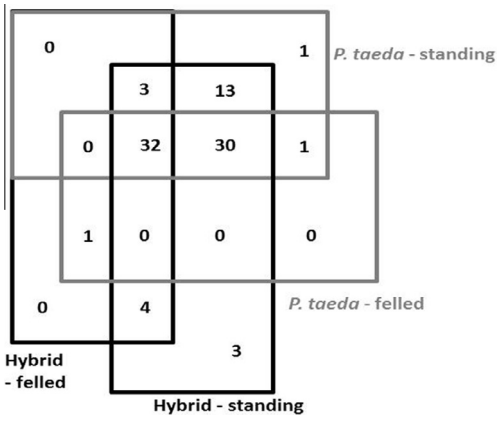

Fig. 8. Number of compounds detected using GC–MS in felled and standing *Pinus taeda* and hybrid pine; intersection of rectangles shows the number of compounds in common between groups.

4. Discussion

The results of this study suggest poorer survival of *Deladenus siricidicola* nematodes in the novel PEE × PCH hybrid host compared with the known host *P. taeda*. Nematodes were never recovered from standing hybrid pine, and nematode survival was lower in hybrid felled trees and billets stored in common conditions compared with *P. taeda*. We identified physical and chemical differences between the two taxa and here consider the potential impact of these in influencing the observed patterns of survival and performance of both nematodes and fungus.

Some physical sapwood properties differed between the taxa studied, and may partly explain the distribution of nematodes observed in the hybrid billets in the laboratory compared with *P. taeda*. Tracheid widths of both pine species were similar to the width of adult nematodes (≈30 μm – Bedding, 1968), so are unlikely to have restricted the spread of nematodes through the timber. The generally larger size of bordered pits in the hybrid may explain the more even spread of the nematodes through these billets compared with that of *P. taeda*, facilitating movement of nematodes between tracheids via bordered pits. We found higher wood density associated with lower nematode success, with hybrids having significantly higher density than *P. taeda*. Likewise, *Bursaphelenchus xylophilus* nematodes have been associated with

Table 2
Retention time, compound ID, and peak area of the seven compounds that best separated the two taxa as standing trees.

Ret. time (min)	Name	RI	Mean area hybrid	Mean area *P. taeda*	*t*-Test
22.104	Dehydroabietic acid (DHA)	2463	23.79 ± 1.2	32.11 ± 15.0	n.s
21.768	Palustic acid	2421	11.55 ± 1.7	3.15 ± 2.2	t = 3.03, P = 0.039
21.016	Torulosol	2273	7.33 ± 1.1	0.774 ± 0.42	t = 5.52, P = 0.005
13.055	8-Camphenemethanol	1513	1.95 ± 0.59	4.74 ± 2.5	n.s
9.205	Verbenone	1218	1.61 ± 0.54	3.74 ± 1.6	n.s
20.233	Unknown (MW 286)	2233	2.62 ± 0.52	0.515 ± 0.099	t = 4.00, P = 0.016
22.507	Resin acid	2514	2.16 ± 1.0	2.46 ± 1.5	n.s.

Table 3
Retention time, compound ID, and peak area of the seven compounds that best separated the two taxa as felled trees.

Ret. time (min)	Name	RI	Mean area hybrid	Mean area *P. taeda*	*t*-Test
22.535	Callistic acid	2518	18.26 ± 18.3	25.17 ± 15.4	n.s.
22.104	Dehydroabietic acid (DHA)	2463	6.58 ± 6.6	7.76 ± 4.0	n.s.
15.527	Methyl 4-hydroxy-2-methoxy-3,5,6-trimethyl benzoate	1735	4.96 ± 3.0	1.92 ± 1.3	n.s.
24.485	Unknown	2786	6.90 ± 1.3	5.43 ± 2.7	n.s.
17.695	2,5-Bis-(3-hydroxypropylamino)-*p*-benzoquinone	1950	5.06 ± 4.8	0.568 ± 0.34	n.s.
13.768	1,2-Longidone	1576	0 ± 0	5.23 ± 2.7	n.s.
21.016	Torulosol	2273	5.77 ± 1.7	1.47 ± 0.53	n.s.
23.106	Methyl 7-oxodehydroabietate	2597	3.54 ± 2.6	2.12 ± 0.56	n.s.

P. pinaster of lower wood density (Rodrigues et al., 2010). Fast drying has also been postulated as a possible explanation for low *D. siricidicola* performance in areas with warm dry winters (Bedding, 2009) but both treatment taxa here did not differ in moisture content at inoculation or after three months. Consistent with Hurley et al.'s (2008) results, we found lower nematode survival in inoculated standing trees compared with felled ones.

Although we identified some differences in chemical profiles of the two taxa, we did not detect any differences in performance of nematodes inoculated onto plates of each, suggesting that these differences in plant chemistry did not directly impact their survival. Propagation of the pine wilt nematode, *B. xylophilus*, is influenced by monoterpene concentrations in *P. massoniana* (Niu et al., 2012) but the taxa investigated in our study did not differ in their concentrations of these.

The most likely explanation for differential nematode performance between the taxa tested here is the lower growth of free-living *D. siricidicola's* food source, *Amylostereum areolatum*, on hybrid substrate. *Amylostereum areolatum* grew more densely on plates containing *P. taeda* than the hybrid substrate, and this may also explain the difference in nematode performance in billets of each under controlled conditions. There was a significant relationship between the number of nematodes recovered in *P. taeda* and the distance from the inoculation point, suggesting that the fungus established and grew from the inoculation point, enabling nematodes to feed and reproduce. In contrast, both the lower numbers and broader distribution of nematodes throughout inoculated hybrid billets suggests that nematodes migrated in search of food, and were unable to reproduce at as high a rate as in *P. taeda*. We were unable to isolate *A. areolatum* from the billets to confirm its distribution because of contamination, but our plate bioassay clearly showed reduced growth of *Amylostereum* on hybrid substrate. In contrast, *D. siricidicola* performed equivalently on plates containing substrate of each taxon, presumably because despite its lower growth on hybrid substrate, the amount of fungus was not limiting to nematode survival. *Deladenus siricidicola* performs better on slower-growing fungus (Morris et al., 2012), and in circumstances where the population of nematodes is very small in the presence of a large *A. aerolatum* biomass, the fungus can invade and kill nematode eggs (Morris and Hajek, 2014). However, we found no evidence of diminished nematode survival on plates with differential growth rates of *A. areolatum* in our study.

The chemical differences we found between taxa may have influenced the growth of *A. areolatum*. For example, the terpenoid torulosol was among the major compounds that differentiated hybrids and *P. taeda* in both standing and felled trees: torulosol and its derivatives have been identified in extracts that show antifungal activity (Meneses et al., 2009). The majority of compounds we detected in our samples were non-volatile oleoresins, composed mostly of monoterpenes and diterpene resin acids, which can present both a physical and chemical defence system against invading pathogens and insects. Growth of a mycangial basidiomyeote fungus was inhibited by several terpenoid compounds tested by Bridges (1987). Similarly, in a study of timber decay resistance, heartwood extracts from *P. elliottii* and *P. caribaea* protected *Pinus radiata* sapwood from decay by individual basidiomycete fungi in the laboratory (Kennedy et al., 1995). In this case, protection against basidiomycete decay did not occur in contact with unsterile soil, where it was proposed that extracts were detoxified by organisms absent from living trees.

If the lower survival of *D. siricidicola* in hybrid taxa is explained by the lower growth of *A. areolatum* in that taxon as occurred in the laboratory, it is possible that the large variation in parasitism rates reported globally (Hurley et al., 2007, 2012; Slippers et al., 2012) could be the result of differential fungal growth on different genotypes, phenotypes or chemotypes of trees. Breeding pines for resistance to Sirex has not been a focus of management because of the success of existing management strategies (Slippers et al., 2015), but it is possible that germplasm could be selected that would promote nematode survival. Further work in identifying these factors is warranted.

Because *S. noctilio* is not yet present in the regions of Australia where hybrid pines are grown, we tested only the survival of the mycetophagous form of the nematode. Further examination of the ability of hybrid pine taxa to support *S. noctilio* development, and parasitism by *D. siricidicola* is required before the wasp reaches these regions.

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