The influence of Amylostereum areolatum diversity and competitive interactions on the fitness of the Sirex parasitic nematode Deladenus siricidicola

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We investigated factors influencing survival of the parasitic nematode Deladenus siricidicola.

Different strains of Amylostereum areolatum did not influence survival of D. siricidicola.

Sapstain fungi competed against A. areolatum, the food source of D. siricidicola.

The sapstain fungus Diplodia pinea competed more aggressively in low moisture conditions.

These competitive interactions could negatively effect the survival of D. siricidicola.

The Sirex noctilio (woodwasp)–Amylostereum areolatum (fungus) complex has caused substantial losses to pine industries in its introduced range. The nematode Deladenus siricidicola that parasitizes S. noctilio and feeds on A. areolatum is widely used as a biological control agent for S. noctilio, but not with consistent success. This variable success could be due to factors that influence the feeding and reproductive ability of the nematode on A. areolatum. We test two main hypotheses that emerge from this prediction. First, we compared the survival of D. siricidicola on the South African field strain and the Australian laboratory strain of A. areolatum, to examine a possible incompatibility between nematode and fungal strain. Second, we examined the competitive interactions of these two A. areolatum strains with two common sapstain fungi, Diplodia pinea and Ophiostoma ips, that occur in S. noctilio infested trees in South Africa. The effect of water potential on the outcome of these fungal interactions was also considered. The data showed that D. siricidicola survives at comparable levels on the two A. areolatum strains. Water potential of the media significantly influenced growth of the fungi and their ability to capture host resource in competitive interactions. D. pinea competed increasingly better against A. areolatum with decreasing water potential. The results suggest that competitive interactions between A. areolatum and sapstain fungi could negatively influence the success of D. siricidicola, especially under conditions of lowered water potential.

1. Introduction

Amylostereum areolatum Boiden is a Basidiomycete fungus which has a mutualistic symbiotic relationship with siricid woodwasps (Cartwright, 1929; Talbot, 1977; Slippers et al., 2003). This fungus
has been recorded in symbioses with the woodwasp Sirex noctilio Fabricius, Sirex juvenscus (Linnaeus), Sirex nitobei Matsumura and Sirex cyanus Fabricius (Bedding and Ackurst, 1978), and more recently with Sirex edwarssii Bruellé and Sirex nitidus (Nielsen et al., 2009). Of these symbioses, the S. noctilio–A. areolatum association is the best known. This is due to the fact that this insect is an invasive alien pest on Pinus spp. in many southern hemisphere countries where these trees are extensively grown in plantations.

S. noctilio attacks various conifer species, but predominantly Pinus species (Spradbery and Kirk, 1978). S. noctilio and A. areolatum in concert overcome the defences of the host tree and provide an ideal environment for the development of the fungus and wasp larvae (Coutts, 1969; Spradbery, 1973; Talbot, 1977). The fungus benefits by being dispersed by the wasp and introduced into a tree which has been weakened by the phytotoxic mucus injected by the wasp into the tree together with the eggs and fungus. In turn, the fungus is essential for the nutrition of the larvae (Coutts, 1969; Spradbery, 1973; Talbot, 1977).

S. noctilio is native to Eurasia, but has been accidentally introduced into many pine growing countries of the southern hemisphere during the course of the twentieth century (Miller and Clarke, 1933; Gilbert and Miller, 1952; Spradbery and Kirk, 1978; Tribe, 1995; Maderni, 1998; Klasmer et al., 1998; Iede et al., 1998; Hurley et al., 2007). In these countries, the S. noctilio–A. areolatum complex has resulted in major losses in pine plantations (Haugen, 1990; Maderni, 1998; Hurley et al., 2007). Most recently, S. noctilio was detected in North America (Hoebeke et al., 2005; de Groot et al., 2007). The threat that the S. noctilio–A. areolatum complex will pose to pine forests in North America is still uncertain, but Yemshanov et al. (2009) estimated the potential damage in Canada over the next 20 years to be as much as $254 million per year.

The nematode Deladenus (= Beddingia) siricidicola Bedding, parasitic to S. noctilio, was discovered and described in the 1960s (Bedding, 1968). D. siricidicola is extraordinary in having both a parasitic and mycetophagous life-cycle (Bedding, 1972). In the mycetophagous life-cycle, the nematodes feed on A. areolatum. In the vicinity of S. noctilio larvae, the high CO2 and low pH environment stimulates the parasitic life-cycle, where the nematodes parasitize the larvae. The nematodes do not kill the larvae, but develop and reproduce inside the larvae, and sterilize the eggs of the emerging female wasp. Infected female wasps lay nematode-filled eggs into new trees, thus spreading the nematode (Bedding, 1972; Bedding and Iede, 2005). D. siricidicola feeds exclusively on A. areolatum in the mycetophagous life-cycle and can go through many generations in the absence of the parasitic life-cycle (Bedding, 1972; Spradbery and Kirk, 1978). The mycetophagous life-cycle takes approximately 2 weeks while the parasitic life-cycle follows the life-cycle of the wasp, which may vary from one to 3 years depending on the environment (Bedding, 1972).

Although complex, the biology of D. siricidicola makes it an ideal biological control agent for S. noctilio (Bedding and Iede, 2005). The nematode can be mass reared on cultures of A. areolatum in a short period of time and inoculated into S. noctilio-infested trees where they feed on A. areolatum in the tree until they locate S. noctilio larvae. D. siricidicola has been released as a biological control agent in every southern hemisphere country where S. noctilio is a pest, often attaining parasitism levels between 70% and 100% (Bedding and Akhurst, 1974; Iede et al., 1998; Tribe and Cillié, 2004; Carnegie et al., 2005). However, success with D. siricidicola has been variable (Hurley et al., 2007). In particular, inoculations with the nematode in the summer rainfall region of South Africa resulted in less than 10% parasitism (Hurley et al., 2007).

A possible explanation for the limited success of D. siricidicola inoculations is competition between A. areolatum and sapstain fungi in the wood (King, 1966). This hypothesis is based on the fact that competition for resources between fungi living on the same substrate is well known and involves primary resource capture or combat (Cooke and Rayner, 1984; Rayner and Webber, 1986; Boddy, 2000). For the S. noctilio system, sapstain fungi are often present in trees infested by this insect (B.P. Hurley, personal observation), and therefore A. areolatum and these fungi would need to compete for the same resource. Two sapstain fungi common in Pinus sp. in South Africa are Diplodia pinea (Desm.) Kick (formerly Sphaeropsis sapinea (Fr.) Dyko and Sutton) and Ophiostoma ips (Rumb.) Nannf. (Wingfield and Swart, 1994; Zhou et al., 2001). D. pinea is a latent pathogen associated with stress and wounds (Swart and Wingfield, 1991), and is commonly found in trees infested with S. noctilio. Similarly, O. ips is associated with the bark beetle, Orthotomicus eruss (Wollaston), which attacks stressed trees (Tribe, 1992; Zhou et al., 2001) and is also common in trees infested with S. noctilio. The limited spread of A. areolatum as a result of competition with these sapstain fungi could decrease the likelihood that the nematodes will survive until they find a fungal source or S. noctilio larvae.

Water availability (water potential) can influence the outcome of competitive interactions between fungal species (Shearer, 1995; Boddy, 2000). Studies on the competitive abilities of insect-associated fungi have shown that the primary and combative ability of the fungi, as well as water potential, influences the outcome of competitive interactions and can have important consequences for the success of the insect–fungus association (Klepzig and Wilkins, 1997; Klepzig et al., 2004; Bleiker and Six, 2009). The influence of moisture availability on inoculation success with D. siricidicola was suggested by Hurley et al. (2008), who showed that inoculation success was lowest in the drier top section of the tree.

The strain of A. areolatum that occurs together with S. noctilio is another factor that could influence inoculation success with D. siricidicola. Certain strains of A. areolatum from the field in Australia were found to be more preferable for rearing the nematode than others (R.A. Bedding personal communication in Slippers et al. (2001), authors, unpublished results). Further, Slippers et al. (2001) showed differences in the strain of A. areolatum present in South Africa and the strain imported from Australia, which is used to rear the nematode. An incompatibility between nematode and fungal strain could result in reduced nematode reproduction and consequently reduced parasitism of S. noctilio. Furthermore, the A. areolatum strain in South Africa could compete poorly with sapstain fungi compared to A. areolatum strains in other countries.

In this study we tested two hypotheses related to the fungal symbiont of S. noctilio that might influence the success of the nematode in biological control programs. First, we compared the survival and reproduction of different D. siricidicola populations on two different strains of A. areolatum, to determine if specificity on the fungus can influence reproductive fitness of the nematode. Second, we examined the competitive ability of the two A. areolatum strains with the sapstain fungi D. pinea and O. ips. Competitive interactions were examined in terms of primary resource capture and combat, and the influence that water potential has on the outcome.

2. Materials and methods

2.1. Nematode sources and fungal strains

Two strains of A. areolatum were used in this study. The Australian laboratory strain (A. areolatum AUS) was sent from Australia with cultures of D. siricidicola in 2003. This strain has been used for rearing nematodes for field release in South Africa since 2004. The South African field strain (A. areolatum KZN) was isolated from the field in 2007 and represents the fungus accidentally introduced with
**S. noctilio.** The *D. pinea* strain was used isolated from *P. patula* in Vryheid, South Africa, in 2007. The *O. ips* strain was isolated from galleries of *O. eross* on a *Pinus* sp., in Lothair, South Africa, in 2009.

All nematodes used in the study were of the Kamona strain of *D. siricidicola* (*Bedding and Lede, 2005*). Laboratory reared cultures from different nematode populations/sources were used, to examine whether the rearing history of the nematode might influence its survival on a specific *A. areolatum* strain. Four different nematode sources were used, namely BRA, ARG, KZN and KZN2. Two of the sources were from laboratory cultures in Brazil (BRA) and Argentina (ARG). The BRA and ARG sources were obtained in 2006 and have been reared on *A. areolatum* (AUS). The other two nematode sources were the original nematodes imported to South Africa from Australia in 2003, introduced into the field, and retrieved from parasitized wasps. The KZN source was retrieved in 2008 and subsequently grown on *A. areolatum* (AUS). The KZN2 source was retrieved from the field in 2007 and subsequently grown on *A. areolatum* (KZN).

### 2.2. Nematode survival and reproduction assays

For the nematode survival and reproduction assays, Potato Dextrose Agar (PDA) (40 g l⁻¹ potato dextrose extract, 15 g l⁻¹ agar) in 90 mm Petri dishes and wheat-rice medium in 500 ml flasks (84 g wheat, 36 g brown rice, 80 ml water) was used as a growth medium for *A. areolatum*. The flasks containing this medium were autoclaved at 121 °C for 30 min, left to stand for 24 h and then autoclaved again at 134 °C for 15 min. Flask cultures (see *Bedding and lede, 2005*) were established for each of the four nematode sources, using *A. areolatum* (AUS). After approximately 6 weeks, nematodes were rinsed three times from each flask using tap water. Three samples were taken from the nematode sediment to estimate the total number of nematodes present. For each sample, 1 ml was removed from the sediment and diluted in 49 ml of tap water, after which 1 ml was removed from this 50 ml solution and the total number of nematodes in this sample was counted using a Petri dish with 0.5 cm² grids, under a microscope at 20× magnification. The nematode–water solution was agitated before each sample was taken to ensure the suspension was well mixed. The average of the three sample counts was used to calculate an estimate for the number of nematodes in the flask.

PDA plates of either *A. areolatum* (AUS) or *A. areolatum* (KZN), where the fungus had grown over one third of the plate, were used to start the cultures for the nematode assays. Nematodes from each of the four sources were placed on plates with *A. areolatum* (AUS) and plates with *A. areolatum* (KZN). In total there were eight treatments of nematode source and fungal strain combination. Approximately 30,000 nematodes were placed on each plate. The fungus was inoculated on one side and the nematodes were placed on the opposite side of the plate. The nematodes moved towards the fungus and after 12 h two fungus–nematode plugs of approximately 3 × 3 cm were cut out and placed face-down on clean PDA plates. Flasks were kept at 23 ± 2 °C and after 2 weeks, three fungus–nematode plugs of approximately the same size were removed from these new cultures and placed in flasks prepared with a wheat–rice medium. A plug of similar size, containing only the fungus was also placed in the flask, to ensure that the nematodes had sufficient food. After 6 weeks the nematodes were rinsed from the flasks and counted, as described above. For each treatment the 10 flasks which appeared to have the highest number of nematodes were counted.

### 2.3. Fungal growth assays

Growth media at different water potentials used for the fungal growth assays was based on previously published methods (*Whit-
3.2. Fungal growth assays

The growth of the fungi was significantly influenced by time, medium, the fungus species/strain and the interaction between medium and fungus species/strain ($p < 0.0001$). Growth of all four fungi decreased with decreasing water potential (Fig. 1). *Amylostereum areolatum* (KZN) grew significantly faster than *A. areolatum* (AUS) on MEA ($p < 0.05$), but not on −5 and −10 MPa (Fig. 1). *D. pinea* and *O. ips* grew significantly faster than *A. areolatum* (AUS) and *A. areolatum* (KZN) on MEA and −5 MPa ($p < 0.0001$) (Fig. 1). *D. pinea* grew faster than *A. areolatum* (AUS), *A. areolatum* (KZN) and *O. ips* on −10 MPa ($p < 0.05$) (Fig. 1). The growth of *D. pinea* and *O. ips* slowed down and stopped as it came close to the edge of the Petri dishes (Fig. 1).

3.3. Fungal competition assays

The growth of the fungi in the competition assays was significantly influenced by time and medium ($p > 0.0001$). There was no clear difference in the competitive interaction of *A. areolatum* (AUS) and *A. areolatum* (KZN) with the two sapstain fungi, *D. pinea* and *O. ips* (Fig. 2). *D. pinea* captured more primary resource than *A. areolatum* on all media, but competed increasingly better against *A. areolatum* with decreasing water potential (Fig. 2). Despite the great differences in primary resource capture, *D. pinea* did not replace *A. areolatum* on any medium. A barrier was formed between the two fungi on MEA and −5 MPa, with inhibition of hyphal growth at a distance, resulting in a deadlock between the fungi (Fig. 3A–B). On −10 MPa, the growth form of *D. pinea* changed and hyphal growth appeared to stop at the point of contact between the two fungi (Fig. 3C).

The competitive ability of *O. ips* against *A. areolatum* decreased with a decrease in water potential (Fig. 2). On MEA, *O. ips* captured far more primary resource than *A. areolatum* (Figs. 2 and 3D). After 4 weeks a barrier was formed between the fungi, with inhibition at a distance. *O. ips* also captured more primary resource at −5 MPa, but the difference in growth between the two fungi was not as great, and no barrier was observed for the duration of the study (Fig. 3E) *Amylostereum areolatum* captured more primary resource than *O. ips* on −10 MPa (Fig. 2). At −10 MPa, isolates of *O. ips* changed in morphology (Fig. 3F).

The competitive ability of *O. ips* against *D. pinea* decreased with a decrease in water potential (Fig. 2). On MEA, *O. ips* initially captured more primary resource than *D. pinea*. A barrier was formed after 3 weeks, which inhibited the growth of *O. ips* (Fig. 3G). *D. pinea* continued to grow, but at the end of the study there appeared to be a deadlock between the two fungi. This deadlock situation only occurred in some of the interactions between *D. pinea* and *O. ips* and was thus not evident in Fig. 2 which represented the average growth. On −5 and −10 MPa, *D. pinea* captured more primary resource and replaced *O. ips* (secondary resource capture) (Figs. 2 and 3H–I).

4. Discussion

This study examined incompatibility between strains of the nematode *D. siricidicola* and the fungal symbiont *A. areolatum*, and competition between *A. areolatum* and sapstain fungi, as possible reasons for the inconsistent control of *S. noctilio* by *D. siricidicola*. The results did not support the hypothesis of incompatibility between nematode and fungal strains, but did support the hypothesis that competitive interactions between *A. areolatum* and sapstain fungi at different water potentials might be involved in the inconsistent control of *S. noctilio* by *D. siricidicola*. Interactions between the nematode, fungi and water availability should thus be considered in current and future biological control efforts against *S. noctilio*. 

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**Table 1**

*p*-Value table comparing nematodes produced on *A. areolatum* AUS and *A. areolatum* KZN fungus, on four nematode sources (ARG, BRA, KZN, KZN2). The difference in nematode numbers between treatments was not significantly different (NS), significantly different where $p < 0.05$ (**), significantly different where $p < 0.01$ (***), or significantly different where $p < 0.001$ (****). LS mean values for the different treatments are indicated.

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<tr>
<th>A. areolatum AUS</th>
<th>A. areolatum KZN</th>
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<tbody>
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<td>ARG 1.0 × 10⁶</td>
<td>6.8 × 10⁵</td>
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<tr>
<td>BRA 2.0 × 10⁵</td>
<td>8.4 × 10⁵</td>
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<td>KZN 1.3 × 10⁶</td>
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<td>KZN2 1.8 × 10⁵</td>
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Fig. 1. Comparison of the cumulative growth of four fungi, *A. areolatum* AUS, *A. areolatum* KZN, *D. pinea* and *O. ips*, on three media of different water potential, over an 18 day period. Growth curves that did not fit a Gompertz function are indicated with an asterix (**).
D. siricidicola released in South Africa as a biological control agent against S. noctilio was able to survive and reproduce on the South African field strain of A. areolatum, introduced naturally with S. noctilio, at levels comparable to its survival on the genetically different Australian strain (Slippers et al., 2001) used to raise the nematodes. The survival and reproduction of the nematode was also comparable to that of the nematode strain that has been introduced in Brazil and Argentina. The nematodes released in Brazil and Argentina have given parasitism levels of over 70% in their respective countries (Leef et al., 1998; V. Klasmer, personal communication), where they are associated in the field with the same strain of A. areolatum introduced into South Africa with S. noctilio (Slippers et al., 2001). The survival and reproduction of all the nematode sources used for biological control in southern hemisphere

Fig. 2. Comparison of the cumulative growth of four fungi, A. areolatum AUS, A. areolatum KZN, D. pinea and O. ips, competing with each other on three media of different water potential, over a 9 week period. Growth curves that did not fit a Gompertz function are indicated with an asterix (*).
countries on the strain of *A. areolatum* occurring with the wasp under field situations in South Africa and South America was comparable. This indicates that incompatibility between *A. areolatum* (KZN) and the Kamona strain of *D. siricidicola* is an unlikely explanation for the low inoculation success in the summer rainfall areas of South Africa.

The KZN2 nematodes reared on *A. areolatum* KZN for multiple generations did not give rise to higher numbers of nematodes on *A. areolatum* KZN in this study. This suggests that efforts to breed for greater survival and reproduction on the *A. areolatum* (KZN) fungus using the Kamona strain of *D. siricidicola* are unlikely to be successful. It is not clear why the nematodes sourced from Brazil gave rise to significantly higher numbers on *A. areolatum* (AUS) than on *A. areolatum* (KZN), but this result highlights the possibility of increased fitness of specific nematode strains on specific fungal strains. Further investigations to understand the behaviour of nematode strains in different environments, as well as their comparable fitness on different *A. areolatum* strains are clearly warranted.

*A. areolatum* generally grew more slowly than the sapstain fungi *D. pinea* and *O. ips*, on the media used in this study. However, the *S. noctilio* symbiont showed strong defence capabilities and was never replaced by the sapstain fungi. *A. areolatum* defended its food resource through the formation of an antagonistic barrier zone some distance from the competing fungi. Alternatively, the fungus produced a stationary barrier zone of mycelium resistant to invasion, at the point of mycelial contact. These forms of combative interactions are well known in fungi. Boddy (2000) stated that antagonism at a distance, hyphal interference; mycoparasitism and gross mycelial contact were the main forms of combative interactions. The outcomes of these interactions may result in replacement of one fungus with another or a deadlock between the two species, as observed between *A. areolatum* and the sapstain fungi.

The growth rate and competitive ability of the two *A. areolatum* strains in this study was very similar on most media. There were only differences on the media with the highest water potential (MEA), where the South African strain grew faster. Inoculation success of *D. siricidicola* is particularly poor at low moisture availability (Hurley et al., 2008), but there were no significant differences in competitive ability of the two *A. areolatum* strains on media with lower water potentials. These results indicate that the difference in the competitive ability of the two *A. areolatum* strains is unlikely to explain the low levels of inoculation success in the summer rainfall areas of South Africa.

Reduced water potential decreased the growth of all of the fungi considered in this study, including the *S. noctilio* symbiont, *A. areolatum*, and the sapstain fungi that it encounters in woodwasp-

![Examples of competitive interactions between the fungi *A. areolatum* AUS, *A. areolatum* KZN, *D. pinea* and *O. ips*. A–C = *D. pinea* (left) competing with *A. areolatum* AUS (right) on MEA, −5 and −10 MPa medium, respectively. D–F = *D. pinea* (left) competing with *A. areolatum* KZN (right) on MEA, −5 and −10 MPa medium, respectively. G–I = *D. pinea* (left) competing with *O. ips* (right) on MEA, −5 and −10 MPa medium, respectively. Pictures were taken at week seven.](image-url)
inoculated nematodes in the tree, as they still have already lost moisture since the attack and at that time D. pinea infection is likely to be well established. This could decrease the survival of the inoculated nematodes in the tree, as they still need to locate the areas of A. areolatum establishment.

The suggestion that competitive interactions between sapstain fungi and A. areolatum could have greater significance on inoculation success than on natural parasitism of D. siricidicola is supported by other data. Although high levels of parasitism have been obtained in South America, results have been variable, and Hurley et al. (2007) suggested that inoculation success is low, but that natural parasitism levels can be high. Similarly, inoculations in the Cape Province of South Africa originally gave poor results (22.6%), but natural spread of the nematode increased parasitism to 96.1% just 2 years later (Tribe and Cillié, 2004). Recent data from the summer rainfall areas also indicates that natural parasitism is increasing (above 50% in some sites, in the bottom sequence comparisons, and Wubetu Bihon (FABI, South Africa) for providing the isolate of D. pinea, also identified using DNA sequences. We also thank Hardus Hatting (FABI, South Africa) for his assistance with rearing laboratory cultures of D. siricidicola.

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References


Acknowledgments

We thank Wilhelm de Beer (FABI, South Africa) for his assistance in isolating O. ips, Tuan Duong (FABI, South Africa) for confirming the identification of the O. ips isolate based on DNA sequence comparisons, and Wubetu Bihon (FABI, South Africa) for providing the isolate of D. pinea, also identified using DNA sequences. We also thank Hardus Hatting (FABI, South Africa) for his assistance with rearing laboratory cultures of D. siricidicola.

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 D. siricidicola. A. areolatum is likely to be able to grow in the low moisture conditions that the nematodes experience in the summer rainfall area of South Africa (Hurley et al., 2008). The growth under these conditions would be slow, and most of the resource could then be captured by D. pinea, which is uniformly present in Pinus spp. in South Africa (Swart et al., 1985; Wingfield and Swart, 1994). However, A. areolatum would be able to defend its resource, thus securing the food source of the S. noctilio larvae and D. siricidicola, which would have entered the tree naturally with A. areolatum. Artificial inoculations with D. siricidicola occur some time after the tree is attacked by S. noctilio, as symptoms of attack must first be observed (Haugen et al., 1990). These trees would have already lost moisture since the attack and at that time D. pinea infection is likely to be well established. This could decrease the survival of the inoculated nematodes in the tree, as they still need to locate the areas of A. areolatum establishment.

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